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Masters Thesis

Development and Characterization of Recombinant Immunotoxins for Cervical Carcinoma

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Plagiarism Declaration

I, **Marc Henry**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Acknowledgements

All praises go to God as his grace has gotten me through the last few years. May he continue to guide me on my journey through life.

My parents, Michael and Margaret, for always supporting me through this long and hard journey. Thank you for all you love and support and shaping me into the person I am today.

To my extended family, thank you for showing an interest in my work.

Thanks to Prof Stefan Barth, it has been an honour to be a member of the Medical Biotechnology and Immunotherapy Research Unit and here's to many more successes in the future.

Thanks to the members of the MB&I group for always being available for a chat and always being willing to lend a hand when needed.

Thank you to the NRF and UCT for helping me pursue my academic journey through their financial support.

I'd like to dedicate this thesis to my late dad, Michael Henry, he was the reason why I had chosen to pursue this journey to obtain my Master's. He always pushed me to strive for excellence and I shall always remember his motto "Hitch your wagon to a star". Here's to you dad.

Contents

List of Abbreviations

HPV	Human Papilloma Virus
HIV	Human Immunodeficiency virus
scFv	Single chain variable fragment
ADC	Antibody drug conjugate
CIN	Cervical Intraepithelial Neoplasia
HAART	Highly active antiretroviral therapy
TZ	Transformation Zone
pRB	Retinoblastoma-associated protein
LSIL	low grade squamous intraepithelial lesions
HSIL	high grade squamous intraepithelial lesions
EMT	epithelial-mesenchymal transition
SNAI 1	snail family transcriptional repressors 1
SNAI 2	snail family transcriptional repressors 2
TWIST1	twist family bHLH transcription factor 1
CSC’s	Cancer stem cells
IKB	NF-$_k$B kinase complex

IKK	inhibitor of nuclear factor kappa -ß- kinase subunit
ETA	Pseudomonas Exotoxin A immunotoxin
EpCAM	Epithelial cell adhesion molecule
EGF	epidermal growth factor
TACE	tumour-necrosis-factor alpha converting enzyme
PS-2	presenilin 2
FHIL2	Four and one-half LIM domains protein 2
V_H	variable heavy chain
V_L	variable light chain
ACT	Adoptive cell transfer
CAR	Chimeric Antigen Receptor
TM	transmembrane
TCR	T cell receptors
CDR's	complementarity determining regions
mAb	Monoclonal antibody
ICC	Invasive cervical cancer
wt	Wildtype

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Abstract

Cervical cancer is the second most frequent occurring cancer in South African women. Cervical cancer accounts for 41.7% of all cancer in black women and 37% in coloured women. If detected early the disease can be treated, however patients are only diagnosed once the cancer has reached its advanced stages. This poor outcome is due to personal reasons of the patient, lack of screening facilities or lack of education. Cervical cancer is a consequence of Human papillomavirus (HPV) infection. Whereby the virus infects epithelial cells of the cervix and integrates its viral DNA into the host's genome. The viral DNA encodes oncogenes and initiates various pathways within the cells to undergo oncogenesis. During oncogenesis the cells protrude various surface receptors to ensure growth and survival of the cancerous cells. Through many cycles of uncontrolled cell proliferation, the cancer will eventually form a tumour in the cervix, this usually occurs during the advanced stages of cervical cancer. Once the cancer has reached advanced stages current treatment has very limited therapeutic effect. This is due to the cancers' ability to develop resistance against current treatments i.e., chemoresistance which leads to a relapse. Therefore, the demand for novel therapeutics for treating advanced stage cancers are high.

Immunotoxins are fusion proteins that consist of an antibody that binds specifically to the cancer cell connected to a protein toxin capable of killing the cell. Immunotoxins have proven to be a promising alternative to current treatment for cancer. Immunotoxins utilize the ability of antibodies to target cancerous cells without affecting the healthy cells due to certain surface receptors being overexpressed in cancerous cells and not in healthy cells. Studies have shown that cell surface receptors LGR5, EpCAM and CD90 are overexpressed in cervical carcinoma. Full length antibodies have been proven to be less effective when used against tumours whereas, various formats of antibody such as fragment antigen binding (Fab) and single chain variable fragment (scFv) demonstrated greater penetration properties.

Pseudomonas exotoxin A (ETA) is a highly cytotoxic enzyme that modifies elongation factor 2 of the cell. This modification is irreversible and arrests protein synthesis thereby causing cell death. ETA is prone to causing a humoral response within patients. This unwanted effect can be prevented by introducing point mutations into the ETA gene. The point mutations are to reduce the chances of an immune response from occurring whilst maintaining the cytotoxic activity of the wildtype (wt) ETA. This study aims to produce scFv antibodies targeting the

three overexpressed biomarkers found in cervical carcinoma connected to a wt ETA or a mutant variant generated from computer simulation by our collaborating partner Prof Paolo Carloni (Forschungszentrum Julich, Germany) described as de-immunised (dETA).

The recombinant immunotoxins (rITs) were generated by designing a bacterial periplasmic expression plasmid containing ETA or dETA to be fused to a scFv antibody fragment targeting one of the three biomarkers (LGR5, CD90 and EpCAM). The corresponding scFv was cloned into this plasmid before being transformed into *Escherichia coli* (*E. coli) BL*21. The periplasm of *E. coli* BL21 was used to produce the rITs by performing bacterial expression under osmotic stress conditions. The rITs were purified using immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). Purified proteins were characterized by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS PAGE) and western blot analysis. Once expression of full-length proteins was confirmed the rITs were assessed on the cervical carcinoma cell line CaSki for binding and cytotoxicity studies.

The results demonstrate that the periplasm of the *E. coli* BL21 can be utilized to generate complex fusion proteins, this being evidenced by the SDS PAGE and western blot results. Both ETA and dETA rITs exhibited strong binding towards CaSki cells. The cytotoxicity assays indicate that both ETA and dETA rITs are capable of targeted killing. However, ETA seems to demonstrate a more potent response compared to the dETA. This may be due to the computer simulated mutant R456T not being able to fully recover the enzymatic activity of B cell epitope depleted dETA to its original wt activity.

The result of this study showcases that this form of treatment may potentially be effective treatment for patients experiencing advanced stages of cervical cancer. However, in future studies other cervical cancer lines should be assessed and eventually patient samples should be assessed in binding and cytotoxicity studies. Although this dETA variant seems to possess a lower potency compared to wildtype ETA immunogenicity analysis using mouse models to determine whether the dETA variant reduces the humoral response within the host would be essential.

Chapter 1: Introduction

1.1) Impact of cervical cancer on South Africa

The South African female population is 21.3 million, ages 15 and older, who are at risk of developing cervical cancer. Cervical cancer refers to the malignant tumour that occurs at the junction with the squamous epithelial cells of the cervix and columnar epithelial cells of the endocervix of the cervix [177]. Cervical cancer is the second most frequent cancer and one of the most common malignant tumours in South African women. Africa has the highest age-standardised incidence (27,6) and mortality (20,0) rate per 100 000 women in the world, with Southern Africa reporting the highest age standardised incidence rate of cervical cancer (43.1 per 100 000).

Women who are diagnosed with advanced stages of cervical cancer, have limited access to appropriate diagnostics and treatment which results in poor survival outcomes. [145] Overall survival is influenced by multiple factors such as socioeconomic factors, availability of programmes, availability of treatment infrastructure ,stage of diagnosis, and health personnel to give timely and appropriate care and co-morbidities i.e. HIV [145]. There are many young women who suffer from sexual and reproductive health challenges which attribute to the limited access of prevention measures, early diagnosis, and treatment. There are multiple potential risk factors that contribute towards the development of cervical cancer. These include parity, gross domestic product (GDP), rural population, access, attitude and beliefs [145].

1.1.1) Parity

A high frequency of unprotected sex results in a greater exposure to HPV thereby increasing the risk of developing cervical cancer. Late-stage cervical cancer diagnosis may result from a high parity due to the lack of contraception, education, and access to screening facilities. High parity also reflects on the inequalities between men and women in access to education, with the negative correlation that exists between women's likelihood of childbearing and the attainment of education being well established [155]. This relationship perpetuates the cycle of high parity and the lack of education amongst generations of women.

1.1.2) Gross domestic product (GDP)

The GDP has been shown to be the only protective model variable against late-stage cervical cancer [155]. Countries with higher GDP are capable of allocating sufficient funds to healthcare sectors. These funds help healthcare sectors with prevention strategies such as screening programmes, education and provide timely and effective treatments for patients in early stages. Therefore, due to low GDP, high incidences of unsafe sex may occur due to lack of sex education and lack of intervention programmes. In addition, gender inequity leaves women unable to negotiate with their partners for the implementation of appropriate protective mechanisms.

1.1.3) Rural population

Large rural communities may have less access to healthcare and limited education on the causes of cervical cancer. This lack of cancer awareness results in a greater likelihood of patients developing late-stage cervical cancer. A study in Cape Town discussed the influencing factors of women's willingness to undergo cervical cancer screening demonstrating that it contributed towards the high number of late-stage cervical cancer in South Africa. However, there are a variety of factors that influence the women's willingness to be screened for cervical cancer including accessibility, costs, waiting time and quality of services as major barriers to routine screening [104].

1.1.4) Access

Mosavel *et al* (2009) indicated that 59% of women had no reason preventing them being screened for cervical cancer, another 16% cited personal reasons e.g., fear or embarrassment and approximately 12% identified the waiting time of 10 years between pap smears as preventing them from going for screening. Furthermore, 9% of participants identified access to healthcare, affordability as well as family reasons such as childcare as major contributing factors.

1.1.5) Attitude and Beliefs

More than half (52%) had agreed with the following statement "If I am meant to get cervical cancer, I will get it – that is fate". The study only reported (17%) of the participants are afraid of clinics / hospitals and that it is embarrassing to get a pap smear. The study also indicated people who claimed it to be embarrassing preferred not to know if they have cervical cancer.

This attitude towards cervical cancer screening was prevalent in the black and coloured communities.

High HIV prevalence increase the risk of contracting HPV due to the congruent transmission patterns and increases the chance of developing cervical cancer due to the patients compromised immune system [155]. South Africa has a high HIV burden, which has a high priority for management resources. This decreases the number of resources (funds, infrastructure and human resources) available for cervical cancer awareness and screening programs. This lack of resources contributes towards the diagnosis of late-stage cervical cancer. Symptoms of cervical concern may be mistaken for HIV infections.

1.2) Relationship between HIV and Cervical cancer

Almost all cases of cervical cancer are caused by oncogenic strains of HPV [177]. For the cervical cancer to develop, an infection of the cervical epithelium by high-risk HPV (HR-HPV) needs to occur. HR-HPV infection may progress from precancerous cervical lesions including low grade squamous intraepithelial lesions (LSIL) and high grade squamous intraepithelial lesions (HSIL) to invasive cervical [89]. Infection with HR-HPV is necessary to cause cervical cancer, however there are other cofactors that contribute to development of cervical cancer e.g., HIV coinfection.

South Africa has the largest HIV burden in the world, whom 60% are women [70]. Women infected with HIV have a higher rate of contracting HR-HPV strains, this is due to their compromised immune system where HIV positive women have a lower likelihood of clearing infections. HIV positive individuals have a greater risk of persistent HPV infection, persistent cervical dysplasia and rapid cancer progression [177]. HIV positive women have a higher risk of developing squamous intraepithelial neoplasia and invasive cervical cancer [101]. A study reported that the presence of squamous intraepithelial lesions in HIV positive women was 18% in comparison to HIV negative women with 5%. HIV positive women are at higher risk of harbouring multiple HPV genotypes [101]. Cervical cancer is harder to treat in woman suffering from HIV. This would reflect in the number of cervical cancer cases in South Africa. Highly active antiretroviral therapy (HAART) has significantly decreased the mortality rate of HIV infected patients. With this treatment patient's life expectancy has significantly increased. The therapy affects the epidemiology of non-AIDS related cancers which plays a role in the evolution of cervical tumours. Before HAART, cancers were

responsible for less than 10% deaths among HIV infected patients [34]. After the implementation of HAART, 28% of HIV patient deaths were caused by neoplastic lesions. Studies speculate that the long-life expectancy of HIV patients undergoing HAART, possess a greater potential to develop cancers.

Cobucci *et al* found an increased risk for developing invasive cervical cancer after undergoing HAART. This increased incidence of HIV positive patients developing cervical cancer may have several explanations. This may be since the female population have a longer life expectancy than men. With that the antiretrovirals does not prevent the development of invasive cervical cancer (ICC) as it was previously theorised. It is more likely that antiretroviral therapy indirectly affects the epidemiology of these cancers because HAART increases the life expectancy of people living with HIV, allowing these cancers to emerge [34].

1.3) Cervical cancer causes

Cancer is caused when a series of mutations in genes of the cell occur. Mutations can influence cellular work processes by affecting the genes normal function. This disruption results in the dysfunction of vital genes e.g., genes responsible for controlling the cell cycle [61]. The most frequently mutated cancer gene is p53 [60]. Mutations in this gene results in the formation of unusual protein which play a role in the cell cycle. Abnormalities in these proteins result in the formation of cancerous cells. Under normal conditions, p53 is responsible for cell division, whereby in various checkpoints of the cell cycle p53 can enable the repair of damaged DNA or initiate cell death if the damage is too severe. Due to these mutations the cancerous cells begin to upregulate certain surface receptors to promote growth and survival [5]. These receptors are overexpressed in cancerous cells which makes it an ideal target as these receptors are not overexpressed in normal cells.

1.3.1) HPV infection

Cancer is a process which involves the uncontrolled proliferation of mutated cells. These cells can invade adjacent and distant tissue by utilizing the circularotry and lymphatic system. HPV infects basal cells and integrates its viral DNA into the host cell genome, which drives oncogenic transformation into cervical cancer stem cells. There are approximately 40 HPV types, persistent HR-HPV infection of the cervix is the main cause of cervical cancer [89].

Infection occurs between the ectocervix and endocervix, this is known as the transformation zone (TZ) [4].

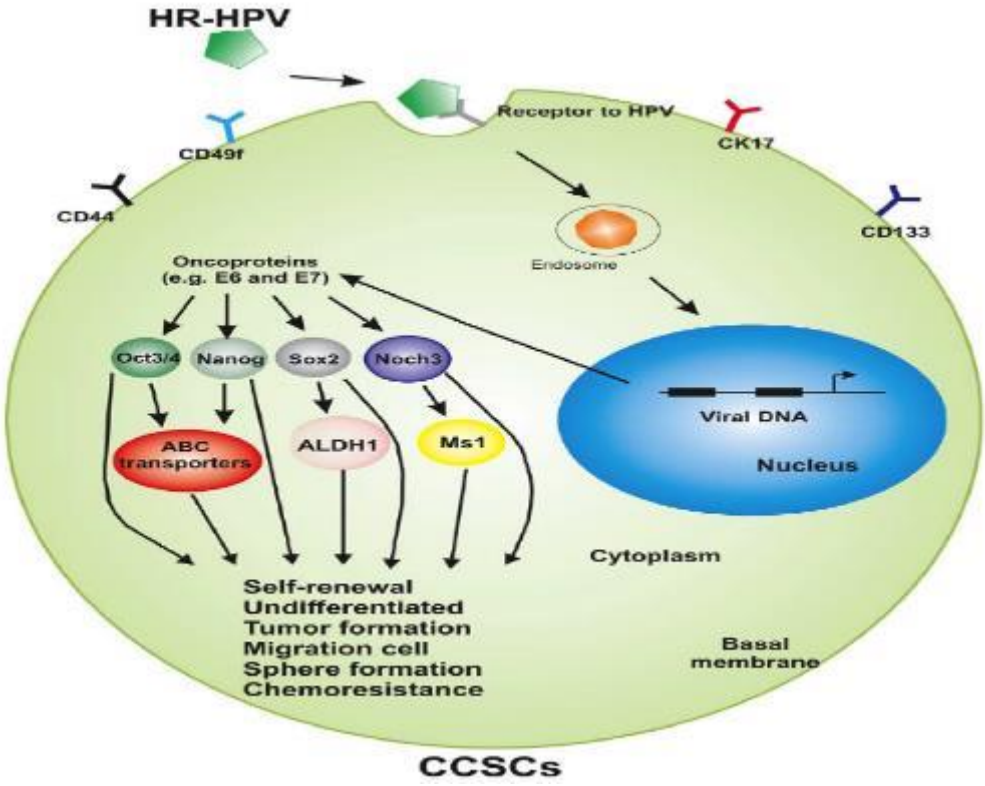

Figure 1: Schematic demonstrating HR-HPV oncoprotein-mediated network regulation of CCSC's [112]. In the TZ cervical epithelium, squamous and columnar cervical neoplasia is initiated by the HR-HPV infection. The virus binding to the surface markers results in the internalization of the virus, once inside the target cell the viral DNA is released and transported to the cell nucleus via the endosomes and lysosomes. This subsequently results in the synthesis of viral oncoproteins. Oncoproteins E7 and E6 promote the proliferation of infected stem cells by inactivating the endogenous tumour suppressor proteins retinoblastoma-associated protein (pRB) and p53 respectively [112]. This inactivation leads to the degradation of pRB and p53 which ultimately results in the termination of Sox2 and Oct3/4 repression whilst increasing the expression levels of Nanog. The HR-HPV oncoproteins increases the expression of genes related to cell self-renewal such as Oct3/4, Nanog, Sox2 and Notch3. Studies have observed that overexpression of stemness-related genes promotes the formation of tumour, inhibits cancer cell apoptosis, cell migration, sphere formation and chemoresistance [112]. This suggests that HR-HPV promotes self-renewal via upregulation of oncogenes Oct3/4, Sox2 and Nanog to maintain cervical cancer stem cell

population in cervical tumours. Overexpression of these oncogenes have reported to promote drug resistance in CSC populations.

1.3.2) Role of viral proteins

The early HPV genes are responsible for the synthesis of viral proteins associated with replication and maintenance of the viral genome as well as late genes that encode proteins associated with forming the viral capsid. The viral proteins are:

- E1: a DNA helicase crucial for viral replication in the host's cell. The E1 viral protein establishes the viral genome as a multicopy episome in the nucleus of cells and can arrange protein-protein and protein-nucleic acid interactions [4].
- E2: The central transcriptional regulator of the virus as it interacts with E1 when DNA replication begins. The E2 viral protein scatters the viral episomal genomes during the division of the infected cells, E2 viral protein mediates the interaction between the viral genome and chromatin adapters proteins at the point of mitotic division. The E2 viral protein indirectly regulates the transcription of E6 an E7 viral proteins. This has many effects on the cell cycle that is responsible for proliferation, differentiation, apoptosis, and senescence.
- E4: this viral protein is found between early replication of HPV genes and expressed during late stages of the infection [4]. The E4 viral protein is associated with the viral material.
- E5: plays a vital role in the productive virus cycle as it has been reported to present oncogenic activities in cultured cells and animals. The E5 viral protein has been reported to modulate the activity of cellular proteins, interacting with targets such as Bax or NF-kB which induce cell proliferation, apoptosis and senescence [4].
- E6: this viral protein is expressed when transformed cells migrate to the spinous cell layer [4]. E6 is responsible for protein synthesis by stimulating rapamycin (mTOR) complex 1, which enhances the 5' mRNA cap translation initiation – complex. The loss of E6 has been reported to result in poor maintenance of the HPV genome due to the failure in p53 degradation. E6 avoids apoptotic responses by binding to procaspase -8 and it is also able to inhibit the responses of interferon response genes [4].

- E7: the viral protein induces HPV viral replication by reprogramming the cellular environment, together with E6, it induces a potent transformation activity in the host cells [4]. The E7 viral protein is responsible for cellular processes such as viral replication, transformation, cell cycle and cell death. The E6 and E7 viral proteins induce degradation of p53 tumour suppressor protein via the Ubiquitin-proteasome pathway, triggering uncontrolled proliferation of the infected cell population [4].
- L1: is the viral protein that has an icosahedral surface on the HPV virions and is the initial point of contact between host cell and virions and is also capable of releasing the viral genome.
- L2: is the viral protein that is involved in initiating in the infection process. L2 helps with HPV DNA encapsulation, and it is a significant component of the late-stage proteins.
- Both L1 and L2 viral proteins are involved in virion assembly in early stages in infection.
- Post infection L2 promotes conformational change on the cell surface to unveil a specific domain of the L2 protein, these are receptors are inaccessible in mature virions to prevent a host immune response.

HPV is a double stranded, circulated DNA that contains noncoding control regions and 8 open reading frames responsible for encoding the viral proteins observed in the “early” and “late” regions [4]. Early stages of viral replication occur in proliferating basal epithelial cells yielding low viral copy numbers. Due to progeny of basal cells the virus replicates and enters spinous cells. The virus matures within the spinous cells, leading to the expression of late capsid proteins and the release of newly formed virions.

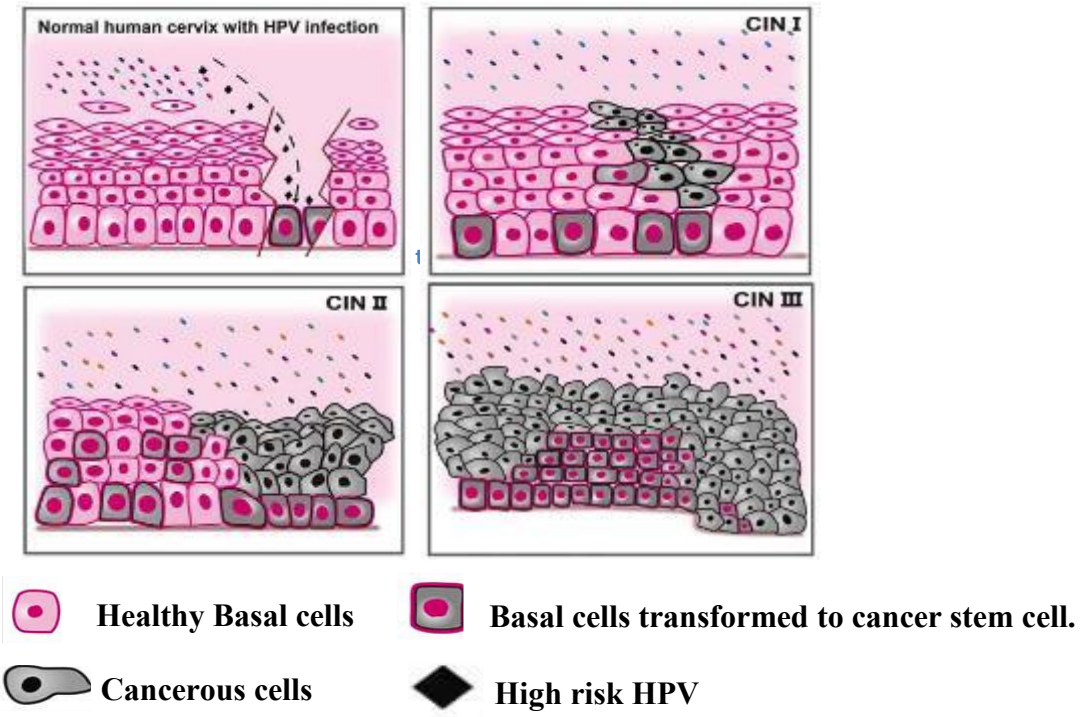

Figure 2: **Schematic demonstrating the factors involved in the progression of cervical cancer [4].** Cervical cancer is initiated by persistent infection with HPV type 16 or type 18 in the basal. When HPV infection occurs, the viral DNA integrates into the host's genome and utilizes the host cells' DNA synthesis machinery. This turns basal cells into cervical cancer stem cells (CCSC). This mechanism allows the virus to escape the hosts' immune response and promotes cell proliferation whilst simultaneously inhibiting the hosts' apoptotic mechanism. As a result, precancerous lesions begin to develop and progress from cervical intraepithelial neoplasia (CIN) grade I – III and eventually cervical carcinoma.

HPV infection may progress from precancerous cervical lesions which include low grade squamous intraepithelial lesions (LSIL) and high grade squamous intraepithelial lesions (HSIL) to invasive cervical cancer [89]. LSIL progresses slowly and mostly asymptomatically, which has a higher fatality rate due to women only discovering the disease in its late stages. Worldwide, high risk HPV type 16 and type 18 is prevalent in (69,4%) of all cervical cancer cases. HSIL accounts for (51,9%), LSIL accounts for (25,8%) and normal cervical cytology accounting for (3.9%). In South Africa, HPV type 16 has the highest prevalence (50,7%) in cervical cancer cases, followed by HPV18 (13.5%). Within 64,2% of cervical cancer cases 33.7% accounts for HSIL, 21.1% accounts for LSIL and 3.2% accounts for normal cytology.

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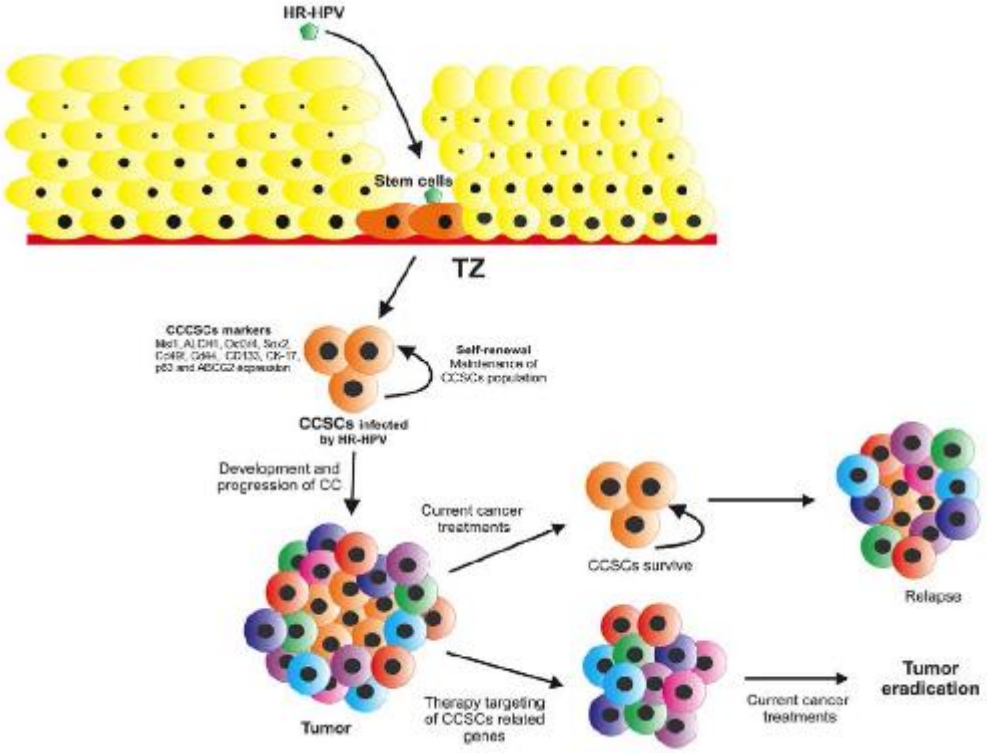


Figure 3: Schematic demonstrating the role of HR-HPV in CCSC's developing drug resistance [112]. HPV E6 and E7 oncoproteins enhance the self-renewal and proliferation of CCSC's by upregulating oncogenes Oct3/4, Sox 2, Nanog and Fgf4.

1.3.3) Cancer stem cells

Another crucial factor in cervical carcinogenesis is epithelial-mesenchymal transition (EMT) [4]. EMT is the process that generates invasive cells, metastasis and is regulated by snail family transcriptional repressors 1 (SNAI 1) and 2 (SNAI 2) and twist family bHLH transcription factor 1 (TWIST1) transcription factors [4]. EMT is a rich in cancer stem cells and its induction promotes metastasis, tumour cell invasion and drug resistance. Cancer stem cells (CSC's) are capable are capable to self-renew or give rise to heterogeneous progeny of cancer cells which drive tumorigenesis. These abilities play an important role in developing and maintaining malignant tumours. CSCs are capable of transporting substances such as drugs through the membrane, which often lead to the recurrence of disease in patients following their treatment. CSCs are also capable of regenerating the tumour and is involved in developing therapeutic resistance, tumour relapse and metastasis [23]. Sun *et al* suggests CSCs may propose a potential target for the elucidation of mechanisms on carcinogenesis and exploration of the targets may lead to novel therapeutics. Not only do stem cells and cancer stem cells share similar characteristic, but they also share the same signalling pathways. These include:

1. Hedgehog signalling pathway.

   This pathway is crucial for the cells self-renewal and the fate of the cell. This pathway is associated with tumorigenesis, development, and progression of certain types of cancer including the maintenance of CSCs [4]. The hedgehog signalling pathway drives the stemness of CSCs via the genetic regulation of Oct4, Sox4 and BMI1 [4]. The hedgehog pathway has been associated with poor outcome in irradiated patients and studies suggest this pathway is involved in the repopulation of cervical cells following chemo radiation.

2. Notch signalling pathway

   This pathway regulates stem cell maintenance, cell fate specification, proliferation, differentiation and angiogenesis [4]. The Notch signalling pathway plays a role in cell-cell communication via transmembrane ligands and receptors. In cervical cancer, the Notch pathway plays several roles depending in the severity of the disease. These roles include:

   1) Maintaining immature epithelium by preventing terminal differentiation.
   2) Increasing Notch expression during the progress of CIN into cervical cancer.
   3) Regulation of Notch signalling pathway by E6 in cervical cancer lines

3. Wnt Signalling pathway.

   This pathway is involved in cell proliferation and differentiation during embryogenesis. The Wnt signalling pathway is the best studied pathway involved in cancer development. CSC's contribution to the maintenance of these cells through the Wnt-ß-catenin signalling pathway [4]. In cervical cancer, apoptosis is induced and tumour growth is inhibited when the Wnt signalling pathway is repressed. However, over activation of the Wnt-ß- catenin signalling pathway is associated with tumorigenesis with HPV infection.

4. NF-$_k$B signalling pathway

   This pathway serves an important role in HPV infected cells. This pathway influences the cancer development by regulating several oncogenic genes [4]. This pathway has 2 routes:

   1) The canonical pathway that depends on the inhibitors of NF-$_k$B kinase complex (IKB).
   2) Non canonical pathway is activated when the homodimer of IKB, inhibitor of nuclear factor kappa -ß- kinase subunit (IKK) is phosphorylated.

5. PI3K/AKt/mTOR signalling pathway.

   PI3K and mTOR are crucial in the proliferation of the cell, angiogenesis, metabolism, differentiation and survival [4]. The pathway is usually activated when mTOR is not correctly regulated under cancer conditions. The PI3K /Akt/ mTOR pathway is essential in regulating self-renewal and maintenance of stemness in stem cells and CSC's.

1.4) Role of LGR5 in Cancer

Leucine-rich repeat containing G protein-couple receptor 5 (LGR5) have been shown to be overexpressed in cervical carcinomas. LGR5 promotes the proliferation of cancerous cells and the formation of tumours via the Wnt/β-catenin pathway. LGR5 modulates canonical Wnt-signalling strength through binding to the ligand R-spondin [179]. By potentiating the Wnt/β catenin signalling pathway, LGR5 can stimulate cancer stem cell proliferation and self-renewal. Cao *et al* indicated a subpopulation of human cervical cancer cell had elevated LGR5 expression possessed an enhanced self-renewal capacity, differentiation potential and tumorigenicity. They also found that tumour sphere forming efficiency increased with the overexpression of LGR5.

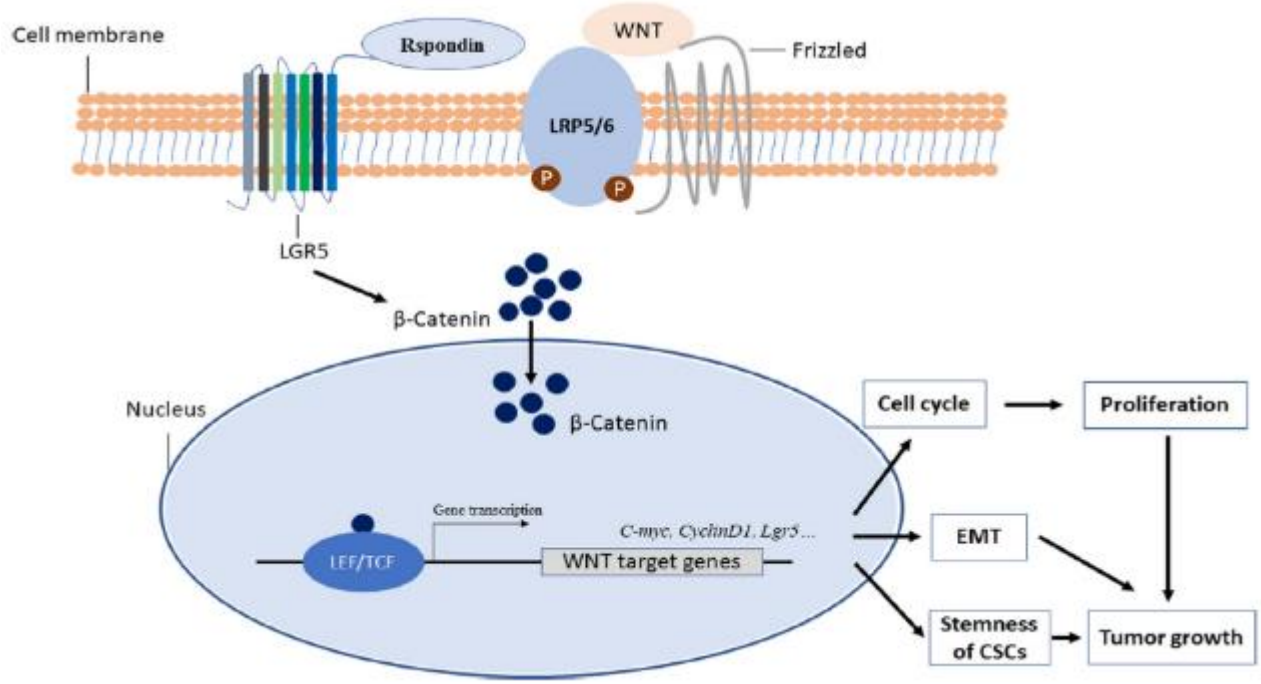


Figure 4: LGR5 role in the Wnt/ß-Catenin pathway [179]. R-spondin activates LGR5, which in turn recruits LRP-frizzled receptor complex which binds to Wnt ligands. This

reinforces Wnt signalling after the phosphorylation of LRP5/6. In the nucleus the accumulation of β-catenin induces the expression of various Wnt target genes (such as C-myc, Cyclin D1 and LGR5). This leads to the binding of TCF/LEF family of transcriptions factors, which results in tumour progression through stimulation of cell proliferation, EMT and stemness maintenance of CSCs [179].

Chen *et al* showed that LGR5 was progressively expressed in cervical carcinogenesis. They found the expression of LGR5 gradually increased from normal cervix (17%) to cancer in situ (15%) and invasive cervical cancer (84%). These findings suggest that LGR5 functions to promote the differentiation and progression of cervical cancers. A cell cycles analysis indicated that an overexpression of LGR5 resulted in a significant increase in the percentage of cells in S phase and a decrease in percentage of G_0/G_1 phase cells. These results suggest that LGR5 progresses cervical cancer by accelerating the cell cycle. LGR5 promotes tumour progression by increasing the number of CSCs in the cervical cancer cell population that are associated with increased cell migration, cell invasion and chemo resistance [23]. These properties prompt the study and development of novel therapeutic targeting LGR5 for treatment of cervical carcinoma. $LGR5^+$ cells have demonstrated to be the cells of origin of tumours, which may provide a feasible approach for effective targeted anti-tumour treatment through targeted elimination of $LGR5^+$ CSC's selectively [179].

1.5) Role of CD90 in cancer

CD90 has been identified as a marker for CSCs in various cancers [170]. CD90 is a surface glycoprotein that is expressed on the cytoplasmic membrane of the cells. Various studies have demonstrated that CD90 is responsible for key functions in cancerous cells such as, regulating the proliferation of cells, angiogenesis and metastasis. CD90 has been shown to influence the biological behaviour and the energy metabolism of cancerous cells.

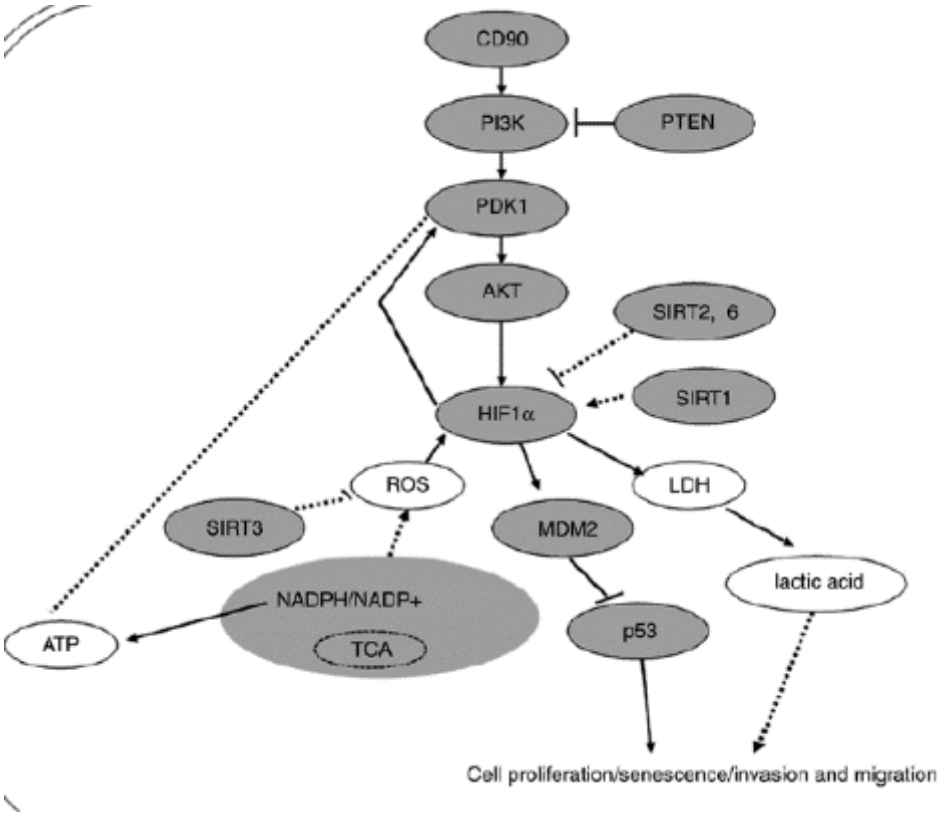


Figure 5: Schematic demonstrating how CD90 upregulates the levels if PI3K, PDK1, AKT, p-AKT-Ser473, HIF-1α and MDM2 and down regulates the levels of p53 and PTEN [54]. The activation of PI3K and AKT signalling pathway is associated with the promotion of cell proliferation in numerous types of malignant tumours. HIF-1α is another key molecule in the PI3K/AKT/HIF-1α signalling pathway, as the dimerization of these subunits (HIF-1α and ß) forms a HIF-1 complex [54]. This complex affects the transcription of crucial genes resulting in tumour cells receiving optimum conditions to maintain growth. Overexpression of CD90 has been correlated with advanced stages of the diseases as well as increased tumour size and decreased overall survival.

Wang *et al* discovered that the Notch and Wnt/ß-catenin signalling pathways play a crucial role in maintaining the self-renewal abilities if CD90.

1.6) Epithelial cell adhesion molecules (EpCAM) role in cancer

Epithelial cell adhesion molecule (EpCAM) is a cell surface glycoprotein that is overexpressed in various cancers of epithelial origin [27]. Chantima *et al* confirmed EpCAM to be a biomarker for cervical cancer as their studies indicated that from cervical cancer tissue collected, all tissues demonstrated an overexpression of EpCAM. EpCAM is homogeneously distributed on the cancer cell surface. EpCAM has been reported to promote cell growth by

regulating classical cyclins (e.g., cyclin D1, Cyclin A and Cyclin E) via the Wnt/ß-catenin signalling pathway [85].

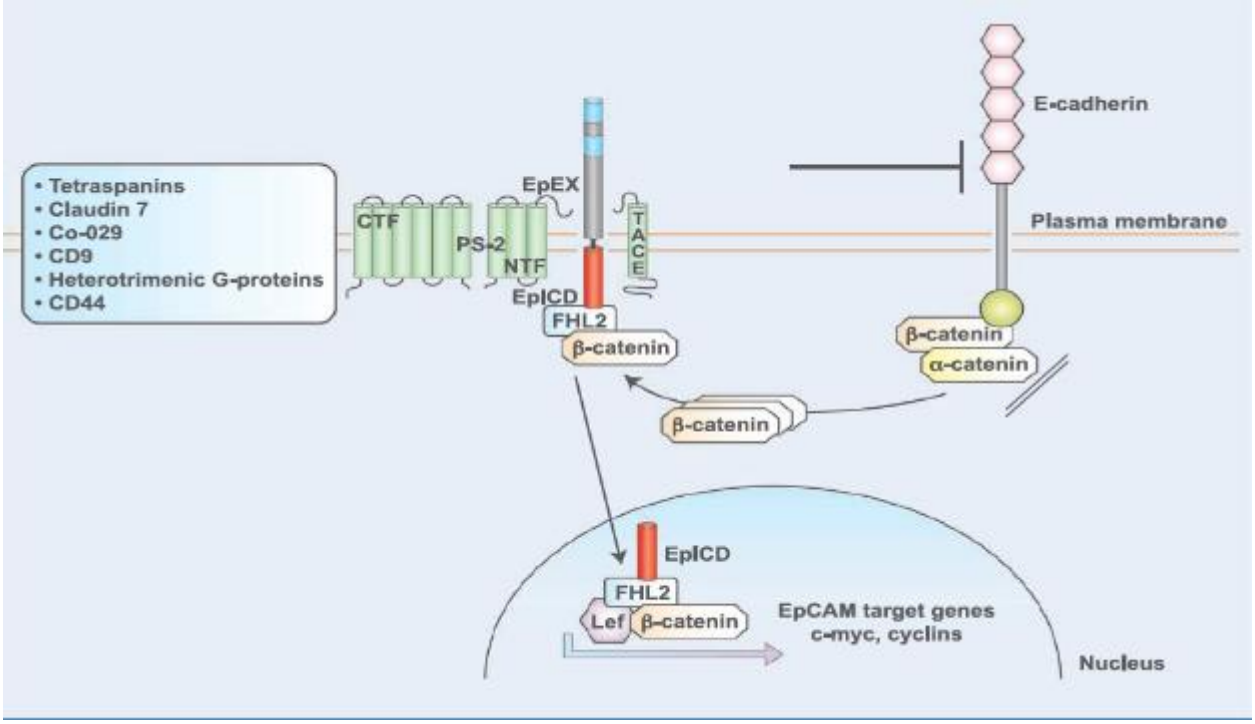


Figure 6: Signalling pathways of EpCAM [105]. EpCAM is comprised of an extracellular domain with epidermal growth factor (EGF) and thyroglobulin repeat-like domains, a single transmembrane domain and a short 26-amino acid intracellular domain called EpICD [106]. Studies show that EpCAM is cleaved by tumour-necrosis-factor alpha converting enzyme (TACE) and a Gamm secretase complex containing presenilin 2 (PS-2) results in a release of EpCAM extracellular domain (EpEX) and EpICD into the cytoplasm. EpICD forms part of a large nuclear complex containing transcriptional regulators ß-catenin and lef, which both are components of the Wnt pathway [106]. Studies have identified Four and one-half LIM domains protein 2 (FHIL2) as a cytosolic interaction partner to EpICD. FHL2 serves as a scaffolding protein for various signalling protein used by EpCAM. It is proposed that interface with E-cadherin-mediated cell adhesion via the PI3 Kinase pathway, EpCAM may increase the level of soluble adaptor ß-catenin which is believed to enhance the nuclear signalling by the Wnt pathway. EpCAM in conjunction with its frequent expression on normal and cancer stem cells and their progeny suggests that EpICD can provide Wnt-like signals in cancerous cells and CSC's.

This was supported by a study that induced overexpression of EpCAM in murine embryonic stem cells. The overexpression of EpCAM allowed for the stabilization of Oct3/4 expression

under various conditions, whereas a reduction expression of EpCAM resulted in a diminished proliferation and expression of stem cell markers. Overexpression of EpCAM is associated with high proliferation and invasion activity in tumour cells as well as with poorer survival in cancer patients [181]. High EpCAM expression is associated with larger tumours and nodal metastasis. In cervical squamous epithelia EpCAM expression increased from low grade to high grade intraepithelial neoplasia and correlated with an increased proliferation, demonstrated in Ki-67 expression [59]. The International Federation of Gynaecology and Obstetrics demonstrated that stage III/IV had higher EpCAM expression in comparison to stage I/II. Suggesting that overexpression of EpCAM correlates to cancer progression.

1.7) Treatments

1.7.1) Current treatments

Surgery is the preferred method of treatment, especially in patients with early stages of the disease. Early stages are also treated with radiotherapy for women who are suitable candidates for surgery. Chemotherapy may or not be administered depending on the risk factors.

Patients suffering from locally advanced cervical cancer (stage IB2 and IVA) have a higher rate of recurrence and worse survival rate [8]. For stage IB2 and IIA1, surgery and radiotherapy have been the go-to treatment as for stage IB3 and IIA2 platinum-based chemo radiotherapy has been the treatment of choice. In severe cases a combination of palliative chemotherapy, carboplatin and paclitaxel have been implemented to reduce symptom and improve quality of life. Bevacizumab has been a treatment recently approved for metastatic/recurrent cervical cancer treatment [8].

Patients with malignant tumours, radiotherapy and chemotherapy are the preferred methods of treatment. Although this method kills most of the diseased cells, this doesn't necessarily "cure" the patient of the cancer.

1.7.2) Targeted therapies

Conventional treatments are nonspecific and risk severe side effects. Cancer cells contain mutant or overexpressed oncogenes on their surfaces [131]. By targeting the cells via their surface proteins, this offers an alternative to the conventional treatments (surgery,

chemotherapy and radiation). This approach eliminates cancer cells whilst leaving the healthy cells unaffected.

1.7.2.1) Antibodies

Antibodies play an important role in the immune system whereby they recognize foreign antigens, neutralize them and elicit an immune response. Antibodies are the common biomolecule used in immunotherapy, due to their multiple formats (IgG, IgA, IgM, IgE and IgD) they able to be engineered to optimize the anti-tumour response.

Antibodies consist of two heavy and two light chains that forms a Y shape. At the end of the Y is the fragment antigen binding (Fab) segment of the antibody, this is responsible for the recognition of the specific antigen. Located at the base of the Y shape antibody is the fragment crystallizable (Fc) region, which mediates the interaction between the antibody and cells of the immune system [182]. From the 5 classes of antibody, IgG is the favourable form to use in antibody therapy. This is because the IgG Fc region interacts with the Fc receptors (FcRs) found on natural killer (NK) cells these include neutrophils, monocytes, dendritic cells and eosinophils [182]. These cells destroy the target cells via specialized functions such as antibody-dependant cellular cytotoxicity (ADCC) and complement-dependant cytotoxicity (CDC). Astrid Fagraeus demonstrated that antibodies can be produced in B cells of the adaptive immune system. Gustav Nossal then proved that a single B cell clone produces antibodies that shares an affinity for the same epitope, this gave rise to what is known as monoclonal antibodies (mAbs). Monoclonal antibodies (mAbs) can be developed to target unique or overexpressed receptors by the cancer cells. These targets mAbs can cause cell death using either a direst mechanism or indirect mechanism.

The direct mechanism involves the blockage of growth factor receptor signalling [182]. An example is Cetuximab, an anti-EGFR mAb that induces apoptosis in tumour cells by blocking ligand binding and receptor dimerization. The indirect mechanism requires the engagement of components of the hosts immune system and causes target cell death via CDC or antibody-dependant cellular phagocytosis (ADCP).

The anti-tumour efficacy of a therapeutic antibody can be improved by linking a cytotoxic molecule to a mAb. This is known as an Antibody drug conjugate (ADC).

1.7.2.2) Antibody drug conjugate (ADC)

An ADC consists of 3 components: the mAb, cytotoxic payload and the linker [69]. Once the mAb bind to the target receptor the ADC can deliver the cytotoxic payload to the target cell via receptor mediated endocytosis. From there the cytotoxin would be released during lysomal degradation and either destroy DNA or inhibit cell division leading to cell death. IgG antibody is mainly used for generating ADCs, however in certain cases the use of full-length antibodies may hinder the therapeutic effect. This is because large antibodies offer poor penetration properties, and the Fc region causes an immune response against the therapeutic antibody. The smallest functional modules of antibodies required for antigen binding are variable fragments. These properties are useful for tumour imaging, due to their small size which improves tumour penetration. Variable fragments are heterodimers of the variable heavy chain (V_H) and the variable light chain (V_L) domains [118]. V_H and V_L of variable fragments can be covalently connected by a peptide linker by fusing the C-terminus of the V_L or V_H. These molecules are known as single chain variable fragments (scFv), these molecules retain the specificity and affinity of original antibody.

1.7.2.3) Immunotoxins

Target therapy can be accomplished by using monoclonal antibodies (mAbs) alone or mAbs armed with radionuclides or toxins [77]. Immunotoxins are proteins that contain a toxin along with an antibody or growth factor that binds specifically to target cells [6]. These toxins originate from plant or bacteria. Most of the protein toxins in clinical development are different types of enzymatic inhibitors of protein synthesis [171]. Bacterial toxins include ETA and diphtheria toxin (DT). Whereas plant derived toxins include ricin, abrin, mistletoe and lectin [84]. Immunotoxins contain 2 components: targeting and killing moieties [171]. Immunotoxins are synthesized by either chemically or genetically conjugating an antibody, fragment to an immunotoxin. Immunotoxins can be fused to the protein binding part of the antibody, known as the variable fragment. Both Fab and scFv are the preferred method of production. The smaller antibody fragments (Fab or scFv) exhibit better pharmacokinetics and maintains full binding properties. Immunotoxins work by binding to the cell surface via internalization to the endosome followed by the translocation to the cytosol and inhibits protein synthesis which leads to cell death.

ETA is a favoured toxin when considering the synthesis of immunotoxins, due to their high potency, expression and purification yields, ease of cloning and low non-specific toxicity

[95]. ETA is a single chain polypeptide consisting of 3 domains: the first domain is responsible for the attachment of the toxin to the cell receptor [147]. The second domain is important for transferring the toxin to the membrane. The third domain contains the enzymatic and catalytic domains, which inhibits protein synthesis which leads to cell death.

There are two important motifs inside exotoxin A. The first of the two motifs, located in the second domain, appears on the toxins external surface and is broken down by the eukaryotic proteases. The second motif is located on the carboxylic end of the toxin and is responsible for retaining the toxin in the endoplasmic reticulum [147]. Both motifs are important for toxicity.

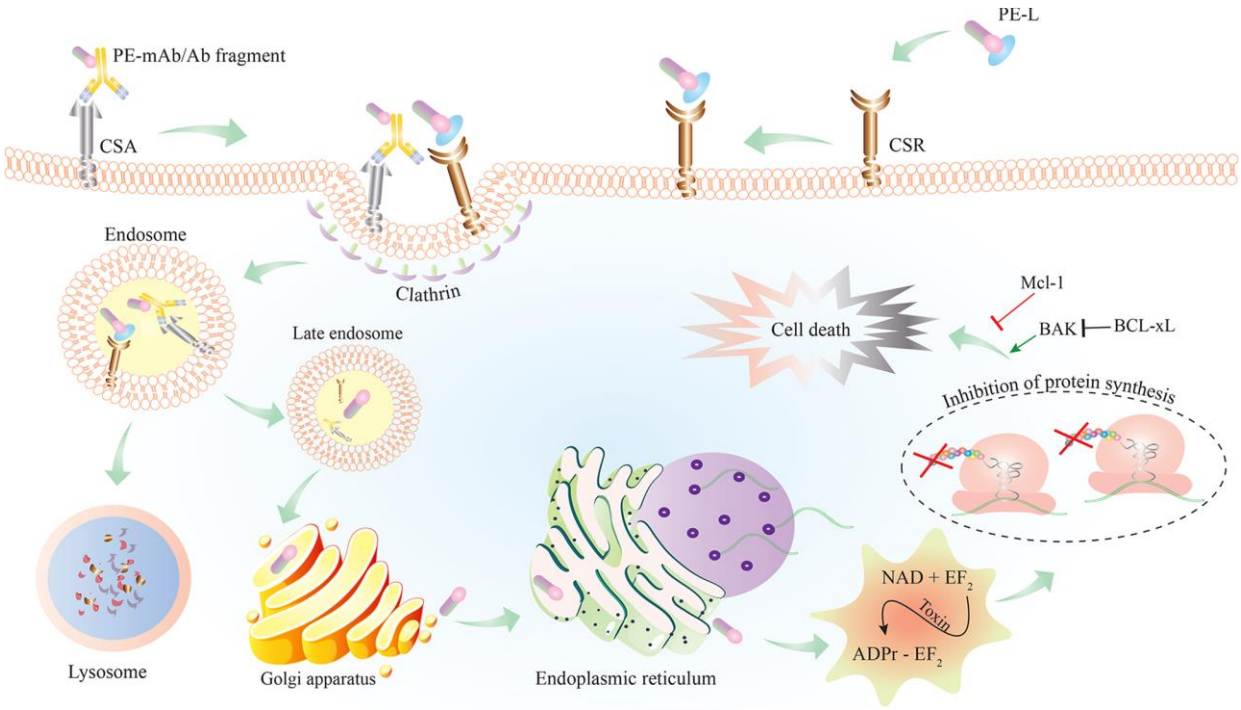


Figure 7: Recombinant Immunotoxins mechanism of killing [62]. An immunotoxin is genetically fused to a scFv antibody that binds to an antigen on a target cell. Once the immunotoxin binds to the target cell, the antigen-immunotoxin complex enters the target cell via endocytosis. The immunotoxin is either localized to an acidified endosome or endoplasmic reticulum (ER) and Golgi apparatus in the cell. Once inside these organelles the linker connecting the toxin and scFv is cleaved. The free toxin catalytically inactivates the protein synthesis machinery of the cell. This is achieved by catalysing ADP ribosylation and inactivating elongation factor 2. This results in the arrest of protein translation and leading to cell death [95].

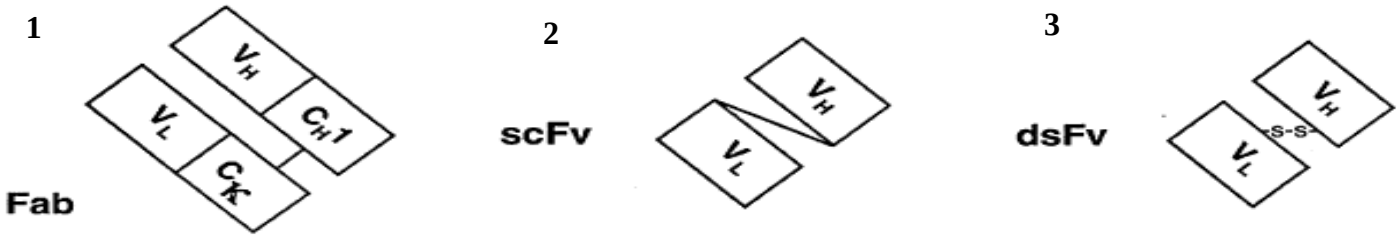


Figure 8: Various Recombinant Immunotoxin antibody formats [75]. ETA can be linked to 3 targeting moieties:

1. Fabs – which are composed of a light chain and heavy chain Fd fragment (V_H and V_L) connected via an interchain disulphide bond.
2. scFv fragments – composed of a variable heavy or variable light chain connected by a peptide linker at the carboxyl terminus.
3. dsFvs – which are disulphide bonds between variable heavy and variable light chains.

1.8) Drawbacks

Despite the promising features ETA brings as an alternative therapeutic, there are a few drawbacks with the use of ETA-based rITs. Firstly, their high immunogenicity in patients, which is the result of ETA being a bacterial toxin. Attempts has been made to reduce the immunogenicity of immunotoxins by deimmunizing the antibody binding domain, modification with macromolecules or structural changes of the ETA domain. Studies have proved that deletions or mutations within the ETA toxin domain has led to a reduced immune response by the patients' immune system. Multiple cycles are prescribed to achieve therapeutic effects, these repeated applications may lead to the activation of the patients' immune system by forming anti-drug antibodies. The toxin has also shown to induce a high humoral response in patients. High concentrations of ETA have led to side effects such as vascular leak syndrome and exhibit non-specific toxicity [83]. To overcome these side effects the following strategies have been applied. Introducing immunosuppressive agents such as monoclonal antibodies and inducing site-directed mutagenesis of the toxin to generate less immunogenic variants. There have been many attempts to reduce the immunogenicity of these ETA immunotoxins by deimmunizing the antibody binding domains, modifying the ETA domain with macromolecules or structural changes to the ETA domain. Several preclinical studies described deletions or mutations within the ETA toxin domain led to the removal of immunodominant B and T-cell epitopes which successfully diminished the immunogenicity of ETA immunotoxins as well as reduced ADA production [99].

SS1P is a rIT composed of a Fv that targets mesothelin linked to 38kDa fragment of ETA [93]. During phase 1 clinical trial 90% of patients treated with SS1P produced neutralizing antibodies in the first cycle. SS1P used in combination with immunosuppressants allowed for more treatment cycles to achieve a major tumour response within the patient. The study demonstrated that the antibodies involved in the immunogenicity response mostly react to the ETA portion of the rIT. Liu *et al* screened a phage display library that contained the Fv portions of antibodies isolated from B-cells of patients who had made anti-SS1P antibodies after treatment with SS1P [93]. The Fvs were used to identify B-cell epitopes and the mutations that suppress these epitopes. This information led to the development of SS1-LO10-R containing a deletion of domain II and six mutations in domain III. This immunotoxin had high cytotoxic activity and reduced immunogenicity however had a short serum half-life. To increase the serum half-life, the Fv was replace by a Fab resulting in an immunotoxin titled RG7787. RG7787 contains seven-point mutations (R427A, R456A , D463A , R467A, R490A , R505A and R538A) , whereas R490A has been described as a general enzyme activating point mutation. By starting a collaboration with the Prof Paolo Carloni (Institute for Advanced Simulation, FZ, Julich), his PhD student Jonas Gossen was simulating possible mutations within the above-mentioned B cell epitopes excluding R490A that would have potential to restore wildtype enzymatic activity. Based on his computer simulation studies, he was postulating R456T and R456C as possible mutations with such a potential.

1.9) Aims and Objectives

1.9.1) Aims

Cervical cancer is the most frequent occurring cancer in women ages between 15 and 44 years. Every year it is estimated that 10 702 women are diagnosed and 5 870 die due to the disease. HR- HPV is responsible for the development of cervical cancer as the infection of epithelial cells in the cervix leads to the integration and translation of oncogenes. These oncogenes promote various pathways within the epithelial cells to initiate oncogenesis.

HIV plays an important role in HPV infection as patients suffering from HIV cannot effectively clear the HPV infection. This contributes to the increased numbers of cervical cancer cases as patients suffering from HIV cannot clear HPV due to their compromised immune system. With conventional treatments the cancer cells can develop resistance towards multiple rounds of therapy. This leads to recurrency and eventually advanced stages

of the cancer, once the patient develops advanced stages of cancer then they have no other than to undergo systemic chemotherapy which has a very limited therapeutic effect.

Immunotoxins are fusion proteins composed of an antibody fragment linked to a protein toxin. The antibody fragment targets the cancer cells specifically and binds to the cancer cell surface receptor. Once bound the protein toxin will be transported to the cytosol of the target cell whereby the toxin will modify eukaryotic elongation factor 2 (eEF-2) leading to termination of protein synthesis. ETA based immunotoxins do have the undesirable trait of eliciting a high humoral response with the patients. This can be negated as studies have shown that point mutations within the toxin domain have successfully reduced the immunogenicity and reduced anti-drug antibody production.

The aim of this study is to generate recombinant immunotoxins (rIT's) targeting the three differentially overexpressed receptors (LGR5, CD90 and EpCAM) in cervical carcinoma each containing either an ETA or deimmunized(dETA) toxin domain. The rITs will be produced within the periplasm of Bl21 *E. coli* and purified using immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). Once characterized the rITs will be labelled and assessed for target specific binding and killing.

This study serves as a proof of concept that complex recombinant proteins can be produced within the periplasm of *E. coli* as well as prove that the dETA can provide the same cytotoxic activity as ETA without eliciting and immune response.

1.9.2) Objectives

The study will investigate the following objectives:

1) Molecular cloning of anti-LGR5(scFv), anti-CD90(scFv) and anti-15EpCAM(scFv) into an ETA or dETA containing backbone.
2) Protein expression, purification, and characterization of full length rITs.
3) Analyse cell binding and internalization properties on cervical cancer cell lines.
4) Analyse the specific killing ability of the rITs on cervical cancer cell lines.

1.9.3) Implication of targeting three different receptors in cervical cancer.

As previously mentioned, cervical cancer is difficult to treat due to the cancers ability to develop resistance to conventional treatment through cancer stem cells. In this study LGR5

and CD90 oncoproteins play an important role in cancer developing resistance towards treatments. Whereas EpCAM is commonly overexpressed in cancerous epithelial cells.

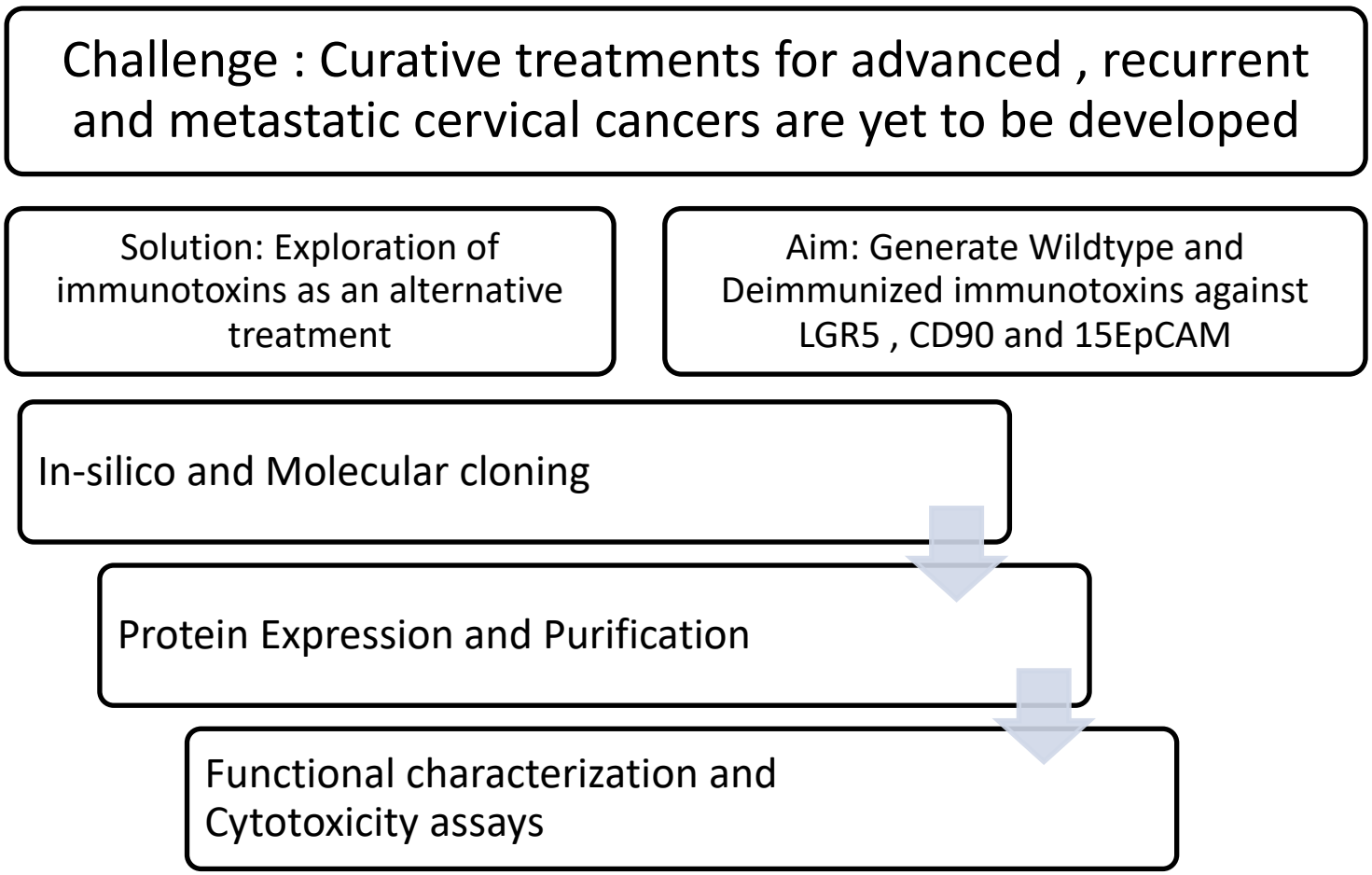


Figure 9: Research workflow: The goal of this research is to investigate the applicability of immunotoxins in advanced cervical cancers. As well as compare the efficiency of the deimmunized immunotoxin against the wildtype immunotoxin.

Chapter 2: Methods and Materials

2.1) Materials

This chapter is providing information on materials and devices used to develop and establish methods, protocols and standard operation procedures at MB&I.

2.1.1) Consumables

All consumables were purchased from the following companies: Bio-Rad (California, USA), Sigma Aldrich (Missouri, USA) , Inquaba Biotechnological industries (Pretoria , South Africa) , New England Biolabs (Massachusetts ,USA) , Qiagen (Hilden , Germany) , Gene script (Piscataway , USA) , Thermofisher Scientific (Waltham , USA) and Beckham Coulter (California ,USA).

2.1.2) Reagents

All buffers and media were prepared in accordance with the manufacturer's instructions using de-ionized water. Media and certain buffers were autoclaved at 121ºC for 20 minutes. Buffers were sterile filtered using a .045µm filter and degassed. Special reagents such as antibiotics (Kanamycin) and Isopropyl-ß-d-1- thiogalactopyranoside (IPTG) were prepared by making stock solutions and stored at -20ºC. The pH of the buffers was adjusted by titration using 1M HCl or 10M NaOH. Pre-made RPMI-1640 culture media were supplemented with 10% Foetal Bovine Serum (FBS) and 1% Penicillin and Streptomycin. The recipes of all buffers are summarised below (table 6).

Table 1: Buffers/Medium used during the study

Reagent	Composition	Concentration
Luria Bertani (LB) broth	Yeast extract	0.5% (w/v)
	Peptone	1% (w/v)
	NaCl	1%(w/v)

LB Agar	Yeast extract Peptone NaCl Agar	0.5%(w/v) 1% (w/v) 1% (w/v) 1.5%(w/v)
SOC	Peptone Yeast extract NaCl KCl $MgCl_2$ $MgSO_4$ Glucose	2% (w/v) 0.5% (w/v) 10mM 25mM 10mM 10mM 20mM
SDS PAGE gel (10%Separating gel)	Acrylamide/Bis-acrylamide (37/1) Tris-HCl (pH8.8) SDS TEMED APS	30% 1.5M, pH8.8 10% 10%
SDS PAGE gel (4% stacking gel)	Acrylamide/Bisacrylamide (37/1) Tris-HCl (pH 6.8) SDS	30% 0.5M, pH6.8 10%

	TEMED APS	 10%
10x SDS Running buffer (pH 8.3)	Tris-Base Glycine SDS	250mM 1.92M 1% (w/v)
10x PBS (pH 7.4)	NaCl KCl Na_2HPO_4 KH_2PO_4	1.37M 27mM 81mM 15mM
10x TAE (pH7.5)	Tris Base Glacial acetic acid EDTA	0.4M 1.14% (v/v) 10mM
Terrific Broth (TB)	Peptone Yeast Extract NaCl	1.0% (w/v) 0.5% (w/v) 1% (w/v)
TBST	Tween 20 Tris NaCl	0.1% (w/v) 20mM 150mM

2.1.3) Equipment

The table below contains the equipment used for the study.

Table 2: Equipment used for this study

Equipment	Manufacturer (model)
Centrifuge	Beckman coulter (Allegra X-30R)
Micro-centrifuge	LMS Co Ltd (MCF-2360)
Weighing balance	Radwag (P5600.R2)
pH meter	Dostmann Electronic (pH50+DHS)
Heating block	Eppendorf (Thermomixer Comfort)
BSLII Cabinet	ESCO Life technologies (Airstream AC2-458)
Cell counter	Bio-rad (TC20)
Electrophoresis	Bio-rad (PowerPacTM HC)
Western Blot	Bio-rad (Trans-Blot Cell)
SpeedVac drier	SP Scientific (miVAC DNA-23050-L00)
Gel imager	Bio-rad (G Box Chemi XL)
Shaking incubator	Yinder Co Ltd (LM-510RD)
Microscope	Bio-rad (Zoe)
Spectrophotometer	Denovix (Denovix Ds-11)

Vortex	LMS Co Ltd (VTX-3000L)
Sonicator	Qsonica L.L.C (Qsonica)
Protein purification equipment	GE Healthcare (AKTA Avant)
Vortex	LMS Co Ltd (VTX-3000L)
Tissue culture incubator	Nuaire (In-VitroCell

2.1.4) Reagents for Molecular Cloning

The table below contains the solutions used during the molecular cloning experiments.

Table 3: Reagents used for Molecular cloning.

Solution	Composition	Concentration
Solution 1	Glucose Tris-HCl (pH8) EDTA (pH8)	50mM 25mM 10mM
Solution 2	SDS NaOH	1% 0.2M
Solution 3	Potassium Acetate Acetic acid	3M
Chloroform: isoamyl alcohol		24:1, v/v
Sodium Acetate		3M

Phenol/Chloroform		1:1, v/v
RNase A		10mg/ml

The restriction enzymes used for this study were *SfiI* , *PvuII* , *NotI* , CutSmart®buffer , T4 DNA ligase and T4 ligase buffer were all supplied by New England Biolabs (USA). The agarose powder was supplied by Sigma-Aldrich (USA). Kit reagents were used in accordance with the manufacturer's guidelines. Table 4 provides the kits used during the molecular cloning procedure.

Table 4: Commercial kits used the study.

Kit	Manufacturer (catalog number)
DNA sample loading dye	New England Biolabs,USA (7025)
QIAquick Gel Extraction kit	Qiagen, Germany (28704)
Zyppy Plasmid Miniprep kit	Zymo Research,USA(D4036)

All newly cloned plasmids were sent to Inqaba Biotechnology Industries (Pretoria,ZA) for sequencing.

2.1.5) Bacteria Strains

Table 5: *E. coli* strains used during this study

Strain	Genotype	Source
Escherichia coli (*E. coli*) DH5α	*supE44 ΔlacU169 (F80 lacZΔM15) hsdR17 recA1*	New England Biolabs,USA

	endA1gyrA96 thi-1 relA1	
Escherichia coli (*E. coli*) BL21(DE3)	*F- ompT hsdSB (rB- mB-) gal dcm- lon-*	New England Biolabs, USA

2.1.6) Cancer cell culture

The cervical cancer cell line was provided by Prof V.Leaner (University of Cape Town). The cancer cell line used for this study was CaSki cervical cancer cell line.

2.1.6a) Reagents for cancer cell culture

Table 6: Reagents used for Cell culture

Reagent	Composition	Concentration	Source
RPMI1640	GlutaMAX	2mM	Gibco 61870
	Sodium pyruvate	3.7g/L	
	Phenol red	15.0 mg/L	
	Fetal Bovine serum	10% v/v	
	Penicillin	100 I.U/ml	
	Streptomycin	100µg/ml	

2.1.7 Protein production and purification buffers

Table 7 shows the reagent and buffers used during the protein production and purification procedure and table 8 shows the commercial reagents used for the characterization of purified protein.

Table 7: Reagents used for Protein production and purification

Buffer	Composition	Concentration
Compatible solutes	D-sorbitol Betaine monohydrate NaCl	500mM 40mM 4%
Binding buffer (pH 8)	Tris-HCl NaCl Imidazole Glycerol	100mM 300mM 150mM 10%
Equilibration Buffer (pH 8)	NaH_2PO_4 NaCl Imidazole	20mM 500mM 40mM
Elution Buffer (pH 8)	NaH_2PO_4 NaCl Imidazole	20mM 500mM 500mM
SEC buffer	PBS	1x

Table8: Commercial reagents used for protein characterization, Functional characterization and Cytotoxicity assays

Reagent/Equipment	Source	Catalogue number
Aqua stain	Bulldog Bio,UK	AS001000
Colour Prestained Protein Standard Broad Range	New England Biolabs, UK	P7719S
PVDF transfer membrane	Roche, CH	03010040001
1-step TMB-Blotting Substrate Solution	Thermo-fisher Scientific, USA	34018
His-Tag primary antibody	Anatech analytical technology, ZA	CST2365S
Goat-anti-rabbit-IgG horadish peroxidase (HRP)	Bio-Rad,USA	1706515
Anti-His PE antibody	R&D systems,USA	IC050P
Mowiol®	Merk,USA	475904
Anti-Fade (Propyl-gallate)	Sigma Alridch,USA	P3130
Cell Proliferation Kit II (XTT)	Roche, CH	52751200

2.2 Methods

2.2.1) *in silico* cloning

The LGR5, CD90 and 15EpCAM ScFv gene sequences were acquired from the patent US9,175,089B2, WO2017/214050A1 and US2015/0017230A1 respectively. To assess the FR and CDR regions. The sequences were then confirmed using Ig BLAST

(https://www.ncbi.nlm.nih.gov/igblast/). Once the sequences were codon optimized, *SfiI* and *NotI* restriction sites were inserted on either side of the scFv gene. SNAP-GENE ® (v.5.0.8, GSL Biotech LLC, USA), was used to insert the anti-LGR5, anti-CD90 and anti-15EpCAM scFvs into a pMT –anti-H22(scFv)-ETA. In the SNAP GENE software, the anti-H22(scFv) gene was replaced with the various scFv genes (LGR5, CD90 and 15EpCAM) to generate pMT-anti-LGR5-ETA, pMT-anti-CD90-ETA, and pMT-anti-15EpCAM-ETA respectively.

The theoretically molecular weight of the recombinant protein was calculated using Expasy translate tool (https://web.expasy.org/translate/). For in silico cloning of deimmunized rITs (dETA), a pUC57 plasmid containing dETA was designed to have *NotI* and *BlpI* restriction sites flanking the dETA gene. These restriction sites are shared by pMT-anti-H22(scFv)-ETA. SNAP-GENE® (v.5.0.8, GSL Biotech LLC, USA) was used to replace the ETA C-terminus with dETA C-terminus.

2.2.2) Molecular cloning

The pMT-anti-LGR5(scFv)-SNAP, pMT-anti-CD90(scFv) and pMT-anti-15EpCAM(scFv)-SNAP plasmids were cultured in LB broth supplemented with Kanamycin (50mg/ml). While the pMT-anti-H22(scFv)-ETA was cultured in LB broth supplemented with Kanamycin(50mg/ml). Both cultures were incubated overnight at 37°C at 180rpm.

2.2.2 a) Plasmid Isolation

The cells were harvested by centrifugation at 4000rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in solution 1 (50mM Glucose, 25mM Tris-HCl (pH8) and 10mM EDTA (pH8)). The resuspended pellet then had solution 2 (1% SDS and 0.2M NaOH) and solution 3 (Potassium Acetate (3M) and Acetic acid) added. Once all solutions were added the reaction was aspirated until the cell debris became visible. The lysed cells were centrifuged at 13000rpm for 10 minutes. The supernatant was then recovered and inoculated with RNaseA (10mg/ml) which was then incubated at 42°C for 30 minutes. Isopropanol was then added and the solution was incubated at room temperature for 10 minutes before being centrifuged at 13000 rpm for 10 minutes. The supernatant was then discarded and the pellet was washed with 70% ethanol before being resuspended in dH2O. The resuspended pellet had Sodium Acetate (3M) and phenol/chloroform (1:1 v/v) added, which was then vortexed and centrifuged for 10 minutes at 13000rpm. After centrifugation, the top phase was collected and had chloroform isoamyl alcohol (24:1 v/v) added. The solution was then centrifuged at 13000rpm for 10 minutes. The top phase was collected and

had 100% ethanol added and incubated at -20°C overnight. The samples were centrifuged at 13000rpm for 20 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol before being allowed to dry in a vacuum centrifuge. The dried pellet was then resuspended in dH2O and quantified.

2.2.2 b) Restriction Digestion

The pMT-anti-LGR5-SNAP, pMT-anti-CD90-SNAP, pMT-anti-15EpCAM-SNAP and pMT-anti-H22(scFv)–ETA plasmids were digested with the *SfiI* (New England Biolabs, #R0123S) and *NotI* (New England Biolabs, #R3189S) restriction enzymes. For the double digest reaction *SfiI* was incubated at 50°C for 3 hours followed by adding *NotI* and incubating at 37°C overnight. Controls remained undigested. The restriction digest products had undergone agarose gel electrophoresis on a 1,2% agarose gel set at 100V for 1 hour.

Table 9:Restriction digestion reaction

Component	Quantity
Plasmid DNA	2µg
Cut Smart Buffer®	5µl
Restriction Enzyme	2µl
Nuclease free water	Made up to 50µl

2.2.2 c) Ligation and Transformation

The bands of interest were excised from the agarose gel and the DNA was extracted using the QI Aquick gel extraction kits (Qiagen, #28704). The DNA extracted from the agarose gel was quantified. The DNA concentration was used to determine the ratio of vector to insert needed for the ligation reaction. The ratios of vector to insert used in the reaction were calculated using the New England Biolabs calculator(https://nebiocalculator.neb.com/#!/ligation).The ratios selected were 1:0 (control), 1:1 and 1:3.

Table 10: Ligation reaction

Component	Quantity
T4 DNA Ligase buffer	2µl
Vector DNA (backbone)	50ng
Insert DNA (1:1 and 1:3)	Required amount
T4 DNA Ligase	1µl
Nuclease free water	Made up to 20µl

The ligation reactions were incubated at 16°C overnight. The ligated products were then transformed into competent DH5α *E.Coli* cells (New England Biolabs, C2987H). The ligated samples and DH5α *E. coli* were mixed were incubated on ice for 30 minutes. The samples were then incubated at 42°C for 60 seconds, which was then followed by incubating on ice for 5 minutes. SOC growth medium (New England Biolabs, #B9020S) was added to the samples and incubated at 37°C for 1 hour. The samples were centrifuged at 10 000 rpm for 2 minutes, followed by discarding 80% of the supernatant. The pellet was then resuspended in the remaining supernatant, which was used to plate on Kanamycin (50mg/ml) supplemented LB agar. The plates were incubated at 37°C overnight.

2.2.2 d) Clone selection and sequencing

Colonies from the 1:3 agar plate were selected and cultured in LB broth supplemented with Kanamycin (50mg/ml) at 37°C overnight at 180 rpm. The plasmids were isolated using a zippy kit (New England Biolabs, # ZR D4019) and the clone plasmid DNA were then used for restriction mapping using *PvuII-HF* (New England Biolabs, R3151S). The reaction was prepared and incubated at 37°C for 60 minutes.

Table 11: Restriction Mapping Reaction

Component	Quantity
Plasmid DNA	1µg
Cut Smart Buffer®	5µl
PvuII Restriction Enzyme	1µl
Nuclease free water	Made up to 20µl

The digested samples undergone agarose gel electrophoresis onto a 1,2% agarose gel set at 100V for 1 hour. Positive clones were then sent to iQaba Biotech for Sanger sequencing.

2.2.3) Bacterial Expression

Positive clones were cultured in supplemented (Kanamycin 50g/ml) LB broth overnight at 37°C at 180rpm. The overnight cultures had its plasmids isolated using the Zippy kit (New England Biolabs, # ZR D4019). The plasmid was then transformed into BL21 *E. coli* cells and incubated on an agar plate supplemented with Kanamycin (50mg/ml) at 37°C overnight. A 50ml starter culture consisting of terrific broth supplemented with Kanamycin (50mg/ml) and a single colony from the agar plate was incubated at 37°C at 180rpm overnight. The starter culture was used to inoculate a 500ml culture. The culture was incubated at 26°C at 180 rpm and the OD600 was measured every hour until the OD600 of the cultures reached 1.6. Once the OD600 has reached 1.6 the culture had compatible solutes (500mM D-sorbitol, 40mM Betaine monohydrate and 4% NaCl) added to the culture and was incubated for 30 minutes at 180rpm. The cultures were then induced with 1mM IPTG and incubated for 16 hours. After 16 hours of incubation the cells were harvested by centrifugation at 4000g at 4°C for 40 minutes. The pellets were collected and weighed. Binding buffer (100mM Tris-HCl, 300mM NaCl , 150mM imidazole and 10% glycerol) was added to the pellet in a ratio of 2:1 (buffer to pellet) and was resuspended and lysed via sonification at the following parameters: 15 seconds on, 15 seconds off at 30% amplitude for 4 minutes. The lysed cells were centrifuged at 24 000g for 30 minutes. The supernatant was collected and filtered using a 0.45-micron syringe filter. The supernatant was purified using IMAC purification. The pellet from the centrifugation was homogenate using a pestle and motor. The homogenised pellet

was centrifuged at 24 000g for 30 minutes. The supernatant was collected was labelled as insoluble fraction.

2.2.4) IMAC purification

The supernatant was loaded into a HisTrap Chelating HP 5ml (Cytiva, #10324336) using an AKTA Avant system. The column was washed with equilibration buffer (20mM NaH_2PO_4, 500mM NaCl and 40mM imidazole) for 15 column volumes. The elution step began with 45% of elution buffer (225mM imidazole) with an increasing linear gradient towards 50% of elution buffer (250mM) for 5 column volumes. The elution then went to 100% of elution buffer (500mM) for 5 column volumes.

2.2.5) Protein Structural Characterization

2.2.5a) SDS PAGE analysis

The eluted fractions were analysed using SDS PAGE. The fractions were mixed with 4x Laemmli Sample buffer (Bio-rad, USA #161.747). Once mixed the fractions were denatured by incubating at 95ºC for 10 minutes. The fractions were then run on a 10% (v/v) SDS gel, where the samples were run at 80V for 20 minutes followed by 120V for 1 hour. Once completed the gels were then visualised by incubating the gel in AquaStain® (Bulldog Bio, USA) overnight.

2.2.5b) Western Blot analysis

The fractions of interest were pooled and concentrated using a 50kDa Amicon® Ultra-15 centrifugal filter (Sigma, USA, UFC901008). The concentrated fractions were then collected and mixed with 4x Laemmli Sample Buffer (Bio-rad, USA, #161747) before being denatured at 95ºC for 10 minutes. The proteins were then run on a 10% SDS gel, once completed the separated proteins were then transferred onto a PVDF (25V for 30minutes). Before the transfer, the membrane was activated by incubating in methanol for 1 minute followed by incubating in distilled water for 1 minute. This process was repeated three times before incubating the membrane in 1x transfer buffer. Following the transfer, the membrane was blocked with fat free milk for 1 hour followed by incubation with anti-His primary rabbit antibody overnight at 4ºC. The following day the membrane was washed with 1x TBST for 5 minutes, which was repeated three times. The membrane was then incubated with goat anti-rabbit horseradish peroxidase antibody for an hour at room temperature. The membrane was

washed three times with 1x TBST before being visualised using 1-step TMB-Blotting Substrate Solution (ThermoFisher, #34018).

2.2.6) Culturing of Cervical cancer cell lines

Cervical cancer cell lines CaSki donated by Prof Leaner (Division of Medical Biochemistry and Structural Biology, UCT). The cell lines were cultured in RPMI media supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and 1% PenStrep (ThermoFisher scientific). The cell cultures were incubated at 37ºC with 5% CO_2. Cells passaged twice a week and counted using a T20 cell counter (Bio-Rad, USA).

2.2.7) Functional Characterization

2.2.7a) Binding analyse using confocal microscopy

The purified protein fractions were pooled and concentrated using a 50kDa Amicon® Ultra-15 centrifugal filter (Sigma, USA, UFC901008) The concentrated proteins(20µg) were conjugated to Anti-His PE antibody (R&D systems, IC050P) in the presence of DTT(5mM). The reaction was incubated at 37ºC for 1 hour in the dark.

The CaSki cell line had $1x10^5$ cells seeded on a coverslip and incubated overnight at 37ºC at 5% CO_2 overnight. The following day, the seeded cells were incubated with Hoechst nuclear stain (1:5000) and 1ml of unsupplemented RPMI for 30 minutes at 37ºC at 5% CO_2. The cells were then washed three times with 1x PBS. The cells were then incubated with the conjugated protein and unsupplemented media for 1 hour at 37ºC. The cells were then washed three times with 1xPBS. The cells were then fixed by incubating in 4% Paraformaldehyde (PFA) at room temperature. Following the incubation, the cover slide was mounted on a slide and viewed using the LSM 880 Airysan confocal microscope (Zeiss) at 40x magnification. Negative controls for these sets of experiments were H22(scFv)-ETA on CaSki cells as the cell line does not express the H22 antigen. For antigen negative controls cells lines HL60 and MCF7 were selected. The HL60 cell line served as an antigen negative control for LGR5 and 15EpCAM, whilst MCF7 served as an antigen negative control for CD90.

2.2.7b) Cell viability assay (XTT)

The CaSki, HL60 and MCF7 cancer cells were treated with rIT's was evaluated by XTT assay using the cell proliferation kit II (XTT)(Roche,CH). The cell lines ($5x10^3$) were seeded

in a 96-well plate and incubated overnight at 37ºC and 5% CO_2. The following day the cells were treated with varying concentrations of rITs and was incubated at 37ºC and 5% CO_2 for 72 hours. After the 72 hours, XTT reagent was added to each well and incubated for 4 hours. As a control Zeocin (100µl/mL) was used to achieve 100% cell killing. Results were analysed by measuring the absorbance of the XTT reagent at 450nm as the measurement filter and 650mn as the reference filter on a spectrophotometer (iMark™ Absorbance reader, Bio-Rad,USA). All experiments performed had five replicates. The absorbance readings were normalized in comparison to the control (untreated and Zeocin) and the results represented as a percentage of cell viability. GraphPrism v.8.0 software was used to calculate the concentration of rIT would achieve 50% cell death (IC_{50}). Negative controls for these sets of experiments were the HL60 and MCF7 cell lines as well as the use of H22(scFv)-ETA. HL60 were used to assess the LGR5 and EpCAM rITs for nonspecific killing. MCF7 were used to assess the CD90 rITs for nonspecific killing. H22(scFv)-ETA was the rIT negative control as the H22 antigen is not expressed in CaSki cell line.

Chapter 3: Results

This section of the thesis will be divided into three sections. The first section covers the proof of concept of expressing protein in the periplasmic space of *E. coli*. The second section covers the generation, functionality and cytotoxicity of ETA-based rITs. The third section covers the generation, functionality and cytotoxicity of dETA-based rITs,

3.1) Proof of concept using pMT-anti-H22(scFv)-ETA

The expression of rITs in the periplasm of *E. coli* under osmotic stress conditions has been well established by Prof Stefan Barth at the University Hospital Aachen, Germany. As a proof of concept for this protocol pMT-anti-H22(scFv)-ETA was used for the first set of protein expression and protein purification experiments. The molecular weight of the rIT was calculated online (https://www.bioinformatics.org/sms/prot_mw.html). The molecular weight of the rIT was 72kDa. The pMT-anti-H22(scFv)-ETA plasmids were transformed into BL21 *E. coli.* Single colony was selected for the bacterial protein expression as mentioned (2.2.3). The cells were harvested and processed for purification (2.2.4). The supernatant was purified using an AKTA Avant (GE.Healthcare). The purified fractions were then concentrated using

an Amicon column followed by size exclusion. The fractions were then analyzed using SDS PAGE and Western Blot.

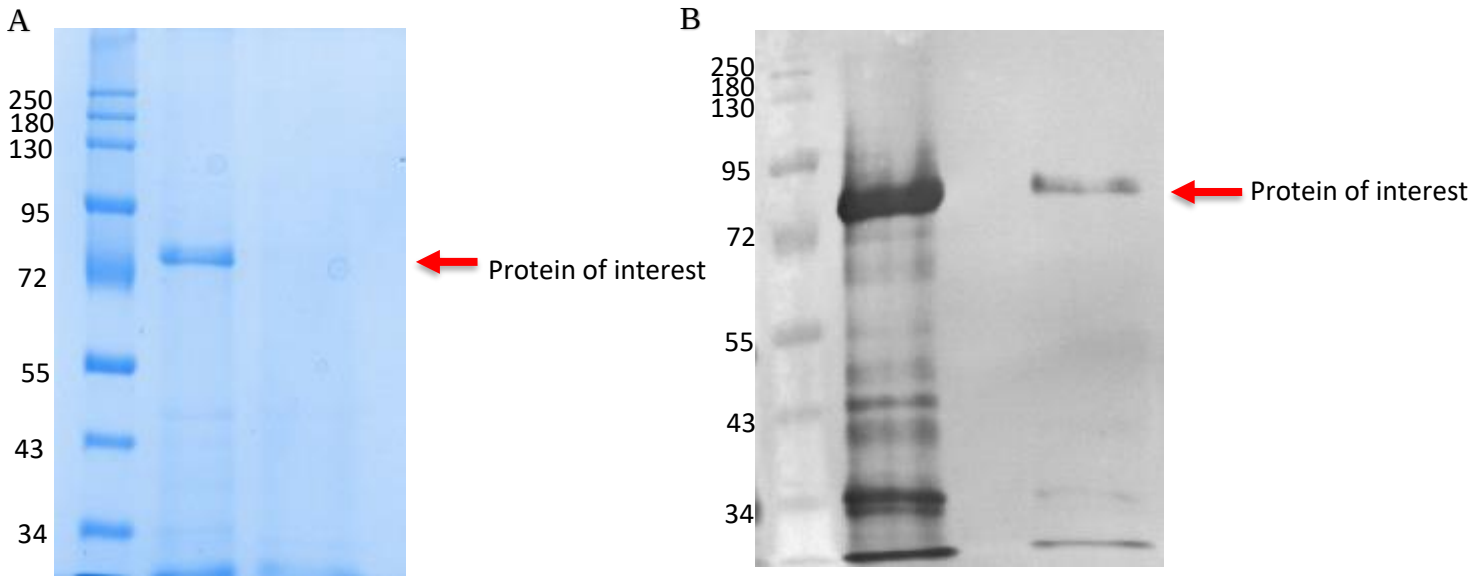


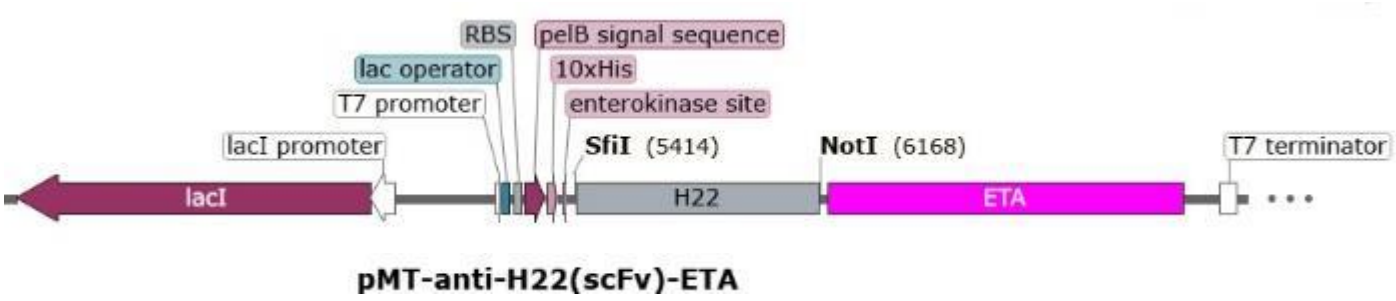
Figure 10: SDS PAGE of purified pMT-anti-H22(scFv)-ETA (A) Western Blot of purified pMT-anti-H22-(scFv)-ETA(B). The purified fractions were first analyzed on SDS PAGE. The fractions containing the protein of interest were pooled and concentrated and used to perform SEC. Lane 1 contains the protein ladder, lane 2 contains post IMAC concentrated fractions and lane 3 contains post SEC concentrated fractions(A). Lane 1 contains the protein ladder, lane 2 contains post IMAC concentrated fractions, lane 3 is blank and lane 4 contains post SEC concentrated fractions (B).

This proved that full-length proteins can be generated in the periplasmic space of BL21 *E. coli*., indicated by the protein in between the 72kDa and 95kDa markers of the protein ladder. The sample after IMAC produced a high yield of protein, however of low purity. Only after SEC, was the pure target protein able to be produced by the expense of the yield.

3.2) *In silico* plasmid design for periplasmic expression of ETA-based rITs

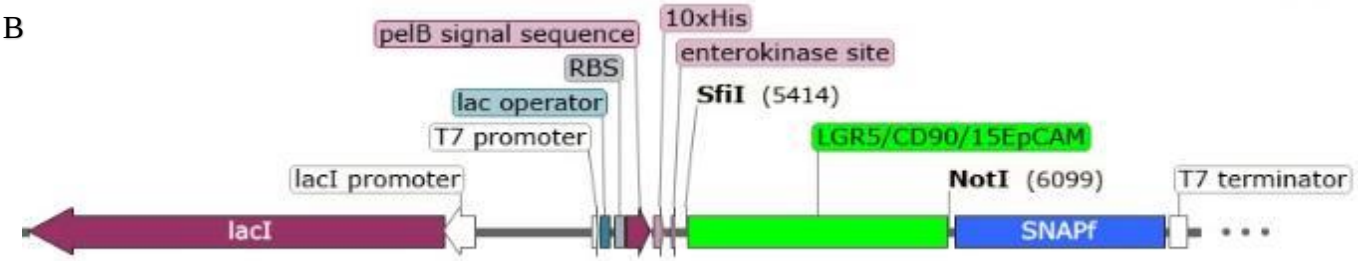
Having proved that rITs are able to be generated in the periplasm of *E. coli*. SnapGene® (v.5.0.8, GSL Biotech LLC, USA) was used to design plasmids for the rITs targeting cervical cancer. Anti-H22(scFv) had *SfiI* and *NotI* flanking it, therefore the plasmids containing the three target scFv (anti-LGR5, anti-CD90 and anti-15EpCAM) were designed to contain the same restriction sites as anti-H22(scFv) in pMT-anti-H22-ETA plasmid.

A



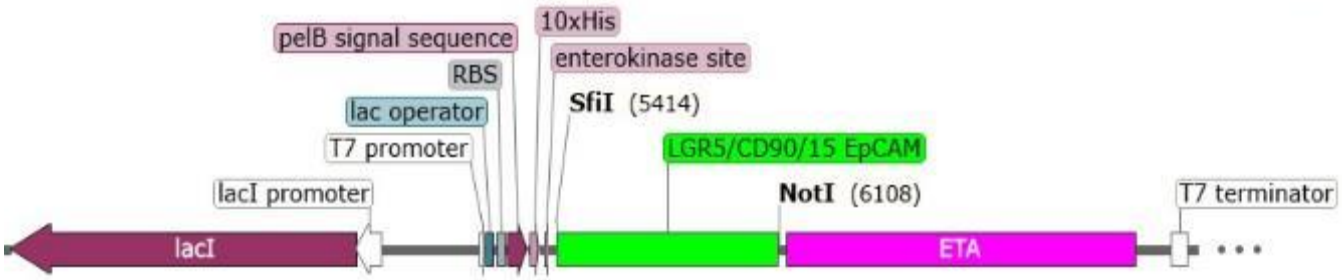
pMT-anti-H22(scFv)-ETA

B



pMT-anti-LGR5/CD90/15EpCAM (scFv)-SNAPf

C



pMT-anti-LGR5/CD90/15EpCAM(scFv)-ETA

Figure 11: Schematic of the *In-silico* design of pMT-anti-H22(scFv)-ETA (A). Schematic of the *In-silico* design of pMT-anti-LGR5/CD90/15EpCAM(scFv)-SNAPf (B). Schematic of the *In-silico* design of pMT-anti-LGR5/CD90/15EpCAM(scFv)-ETA(C). The open reading frame (ORF) of the pMT-vector of periplasmic expression contains a pel B sequence for the transport of the recombinant protein to the periplasmic space. A poly (10x) histidine tag (His-tag) for downstream processes. A kanamycin resistance gene for selective screening.

A lac operator which induces the translation of the rIT. An enterokinase cleavage site (EKS) for post purification tag removal and the C terminus contains ETA.

The scFvs' (anti-LGR5, anti-CD90 and anti-15EpCAM) were able to be replace the anti-H22(scFv) resulting in a new pMT plasmid encoding ETA fused to an scFv targeting either LGR5, CD90 and 15EpCAM. This provided a method for proceeding with the molecular cloning of the wild type rITs

3.3) Molecular cloning of ETA-based rITs

During the *in-silico* process the molecular cloning procedure was able to be simulated. The simulation allowed the study to generate the expected digestion products of the respective plasmids. The plasmids were digested with *SfiI* and *NotI* restriction enzymes and analyzed on a 1,2% agarose gel.

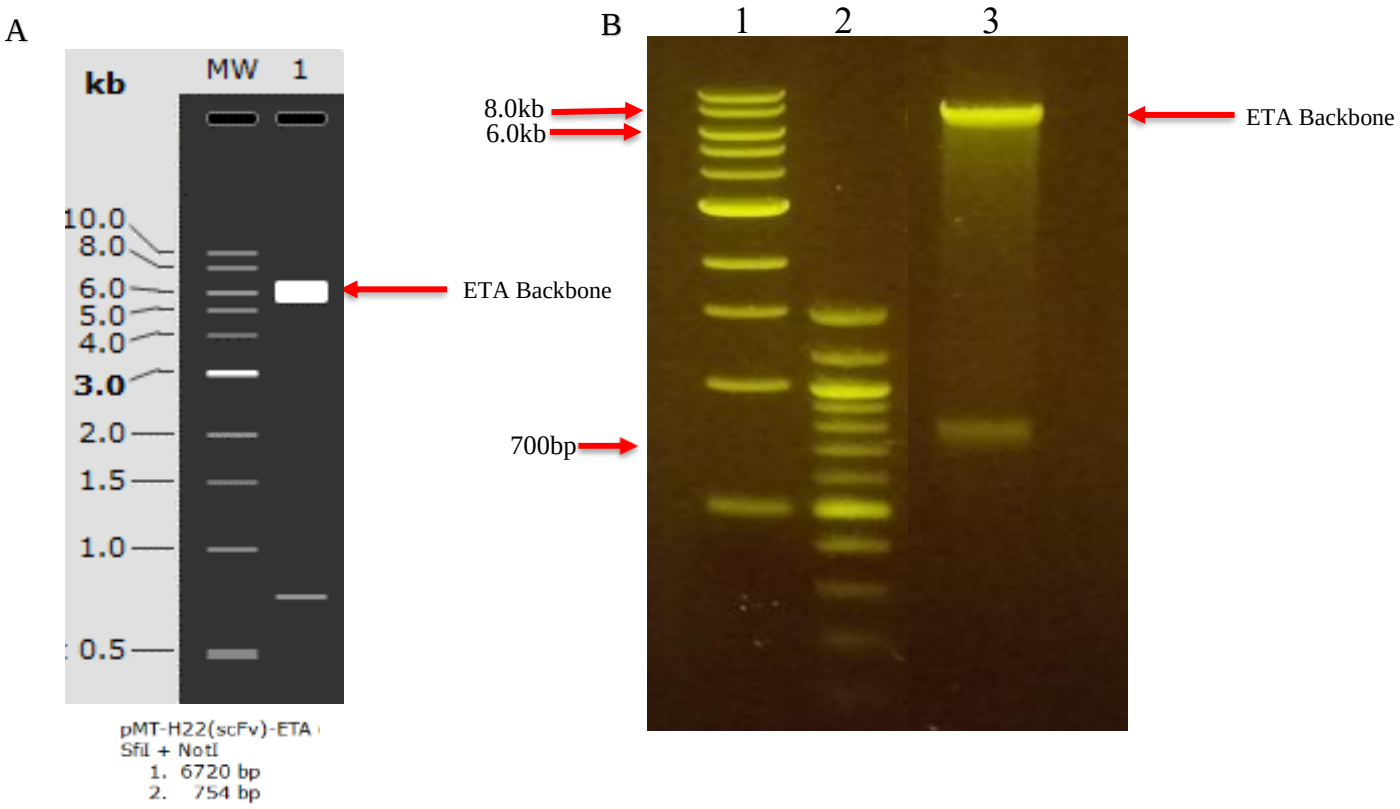


Figure 12: Simulated double digestion product of pMT-anti-H22(scFv)-ETA(A). Lane Mw represents the simulated marker and Lane 1- represents the simulated double digested product of pMT-anti-H22(scFv)-ETA. **Double digested product of pMT-H22-ETA(B)**.

Lane 1 – contains a 1Kb marker, lane 2- contains a 100bp marker and lane 3 contains the double digestion product of pMT-anti-H22(scFv)-ETA.

The double digestion of pMT-anti-H22(scFv)-ETA was successful as the digestion product matched the simulation. This was observed as both the simulation and the double digest produced two bands both at 6723bp and 754bp. The H22(scFv) is represented by the 754bp, this indicates that the band at 6723bp represents the ETA containing backbone. The 6723bp backbone was excised from the gel. The three scFvs were digested using the same combination of restriction enzymes and analyzed on a 1.2% agarose gel. A simulation was performed to calculate the expected size of the target scFvs.

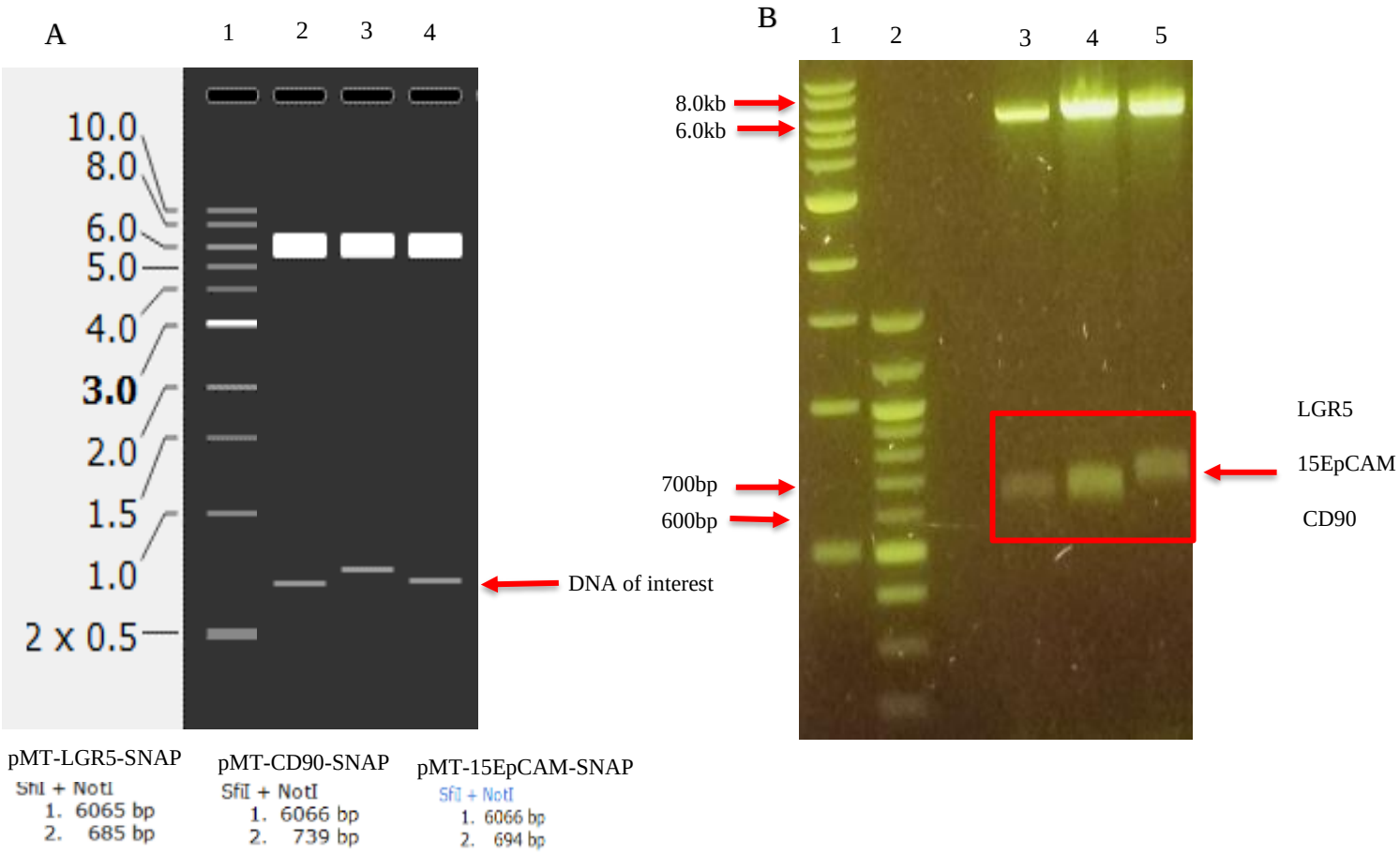


Figure 13. Simulated double digested of pMT-anti-LGR5(scFv)-SNAPf, pMT-anti-CD90(scFv)-SNAPf, and pMT-anti-15EpCAM(scFv)-SNAPf(A). Lane 1 contains the marker, Lane 2 contains simulated double digested product of pMT-anti-LGR5(scFv)-SNAPf, Lane 3 contains simulated double digested product of pMT-anti-CD90(scFv)-SNAPf and Lane 4 contains simulated double digested product of pMT-anti-15EpCAM(scFv)-

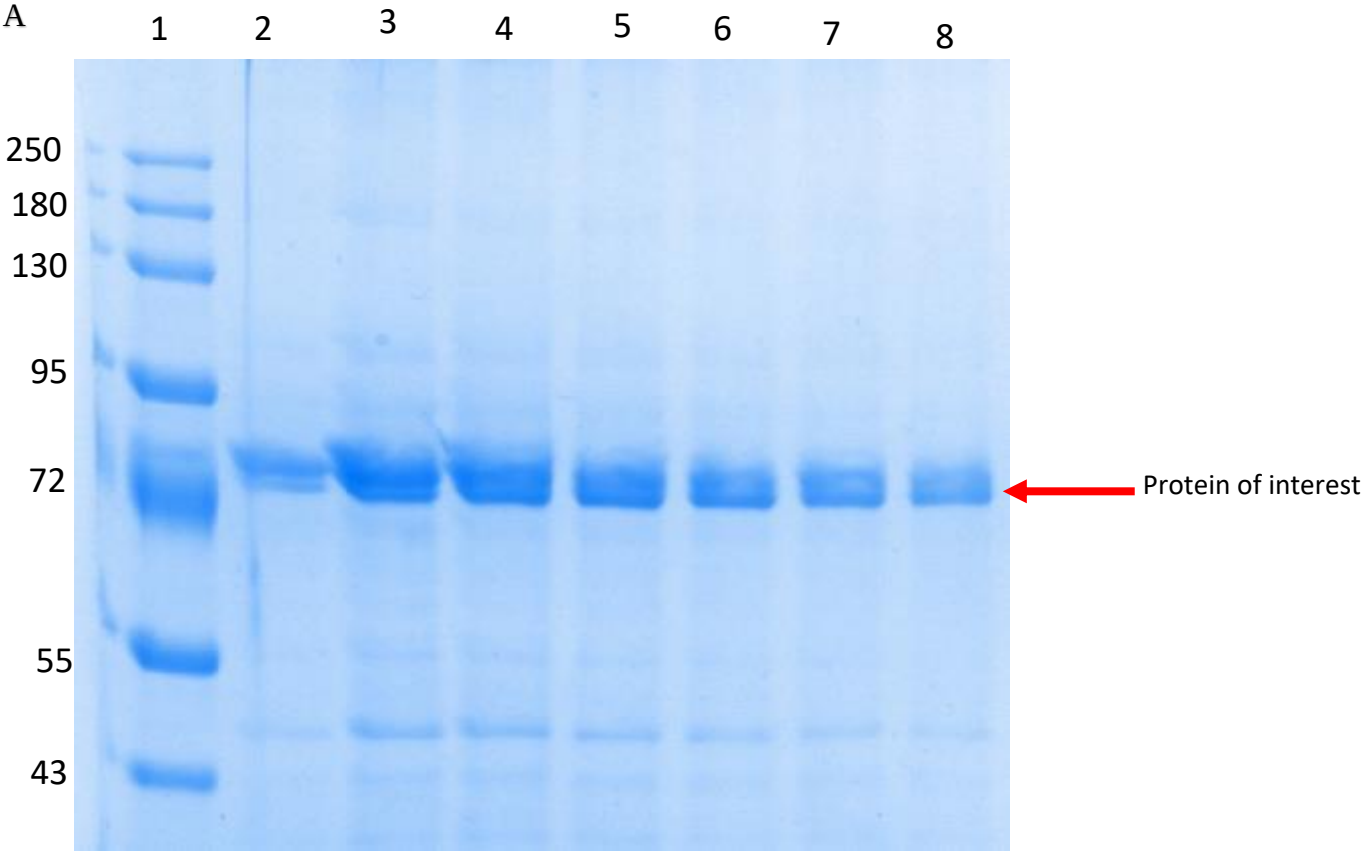
SNAPf. **Double digest product of pMT-anti-LGR5(scFv)-SNAPf, pMT-anti-15EpCAM(scFv)-SNAPf and pMT-anti-CD90(scFv)-SNAPf (B)**. Lane 1 contains a 1Kb marker, lane 2 contains a 100bp marker, lane 3 contains the double digest product of pMT-anti-LGR5(scFv)-SNAPf, lane 4 contains the double digest product of pMT-anti-15EpCAM(scFv)-SNAPf and lane 5 contains the double digest product of pMT-anti-CD90(scFv)-SNAPf. The simulated agarose gel indicates the expected band sizes of the various scFv genes. LGR5(scFv), CD90(scFv) and 15EpCAM(scFv) generated a band of 685bp, 739bp and 694bp respectively. The double digest results match the simulated results as all the double digest reactions produced bands between the 600bp and 700bp marker. These results indicate a successful restriction digestion.

The fragments of interest were excised from the agarose gel and used for a ligation reaction. The insert and backbone DNA were ligated using T4 ligase at various vectors to insert ratios. The ratios used were 1:1 and 1:3. For controls for these experiments were 1:0. These ligation products were then transformed into DH5α *E. coli* cells. Untransformed cells were used as a control to ensure successful transformation. For the control plates the unsupplemented plate had growth whilst the untransformed cell on supplemented plates (Kanamycin 50mg/ml) had no colonies. On the 1:0 plate there were 10 colonies, whereas the 1:1 and 1:3 plates both had >300 colonies. The 1:3 ratio plate had 6 colonies selected and cultured in 3ml of LB broth supplemented with kanamycin (50mg/ml). The plasmids were then isolated using zippy plasmid miniprep. The isolated plasmid had 1µg of DNA used for restriction mapping using *PvuII*. The clones exhibiting a matching profile to the simulation was sent to Inqaba Biotech (Pretoria, South Africa) for Sanger sequencing to confirm successful molecular cloning.

3.4) Protein purification and Characterization

The molecular weight of the rITs were calculated using a Protein Molecular weight calculator (https://www.bioinformatics.org/sms/prot_mw.html). All the rITs had a molecular weight of 72kDa. With the periplasmic protein expression protocol established and the clones confirmed, the plasmids were transformed into BL21 *E. coli*. The BL21 *E. coli* were cultured and were used to perform bacterial protein expression under osmotic stress. The cells were harvested and resuspended before being lysed via sonification. The supernatant was harvested and filtered before undergoing IMAC purification. The IMAC fractions were analyzed on a 10% SDS PAGE.

A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3
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7
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130
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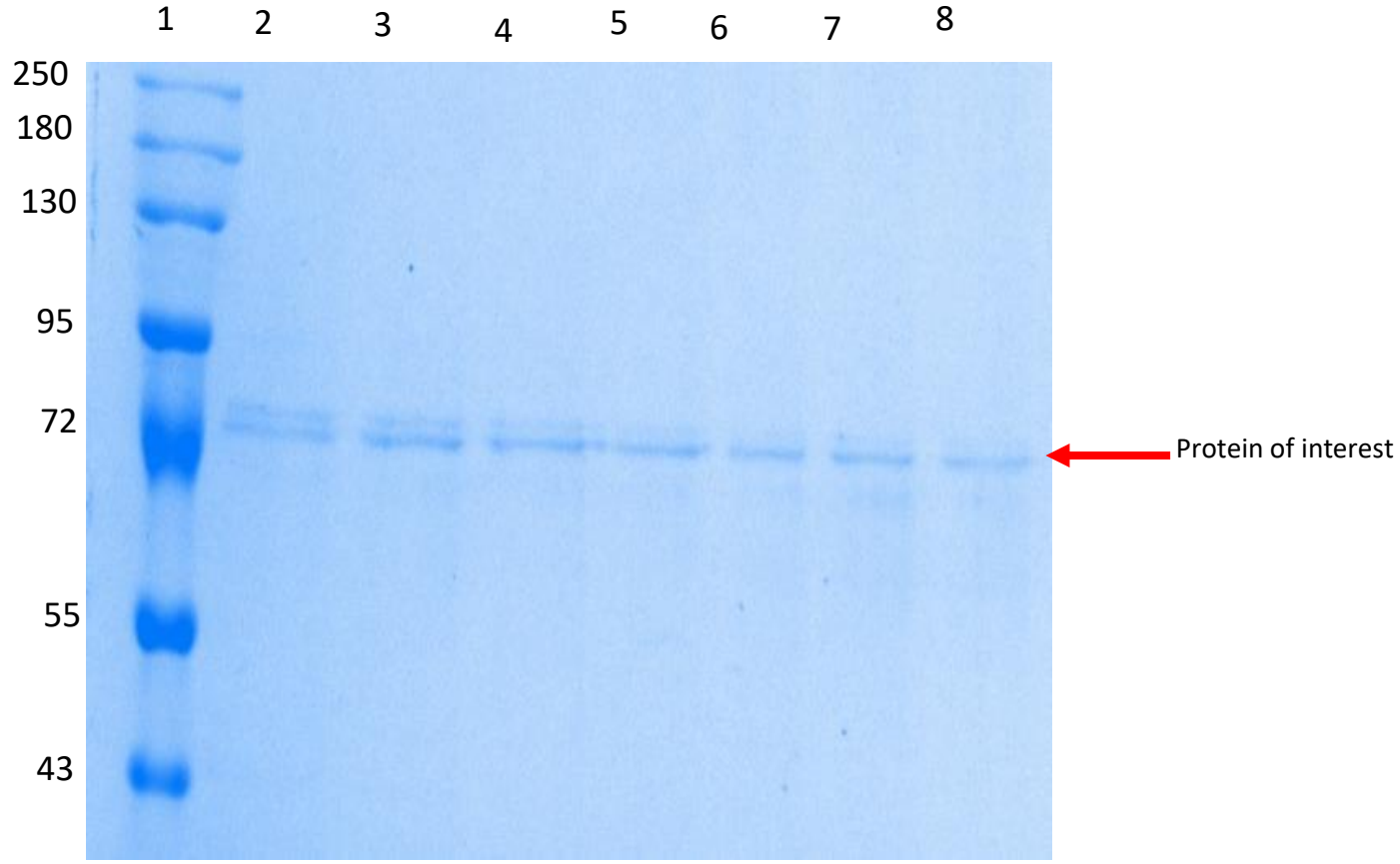
55
43
Protein of interest

B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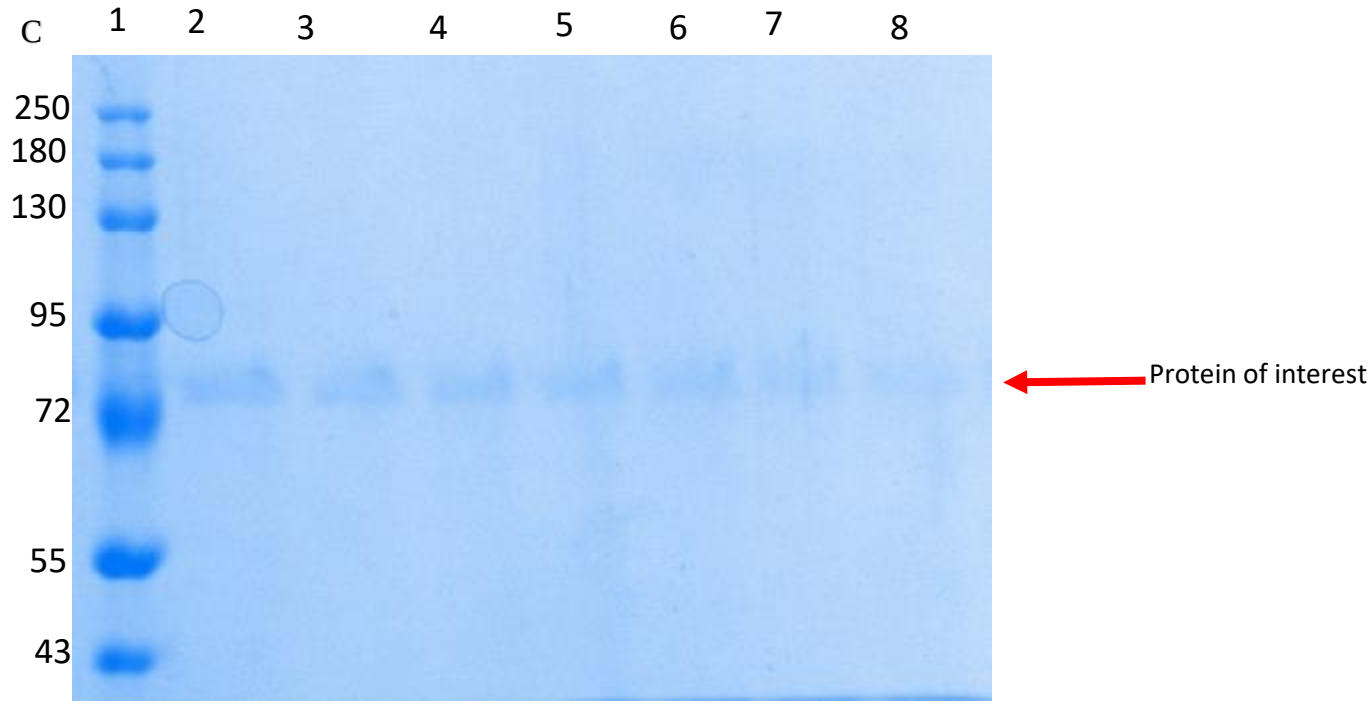
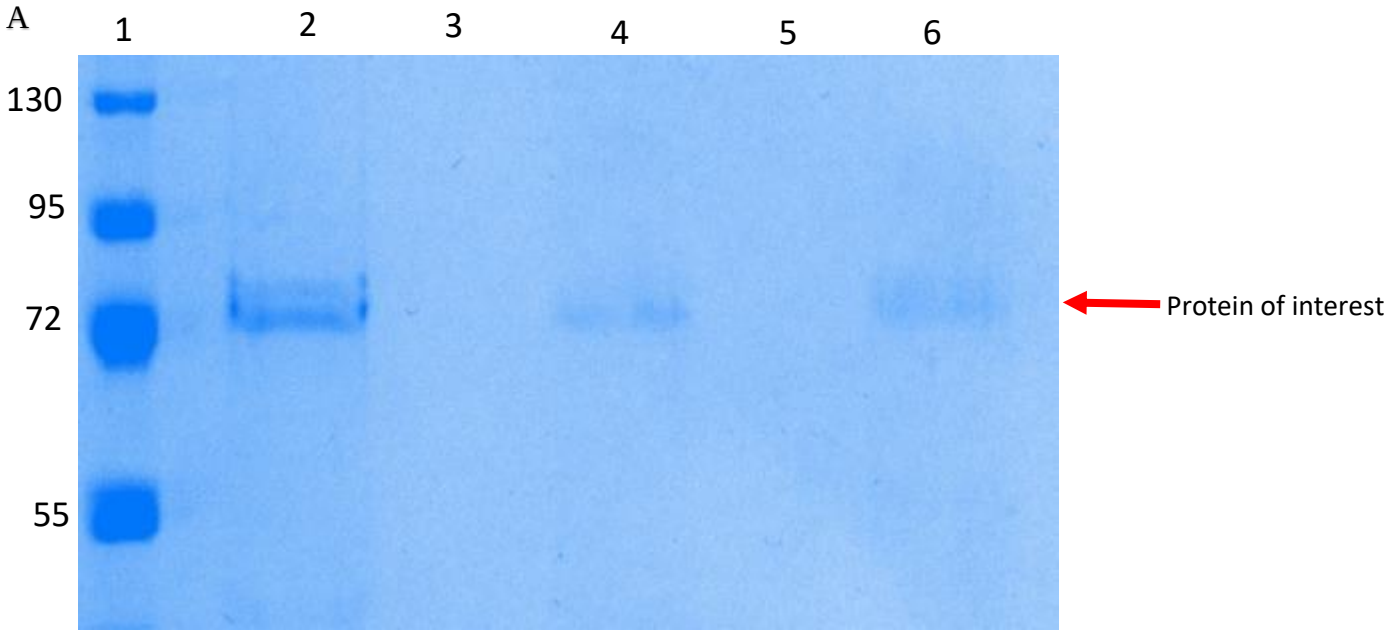


Figure 14: SDS PAGE analysis of IMAC fractions of purified rIT's. IMAC fractions of purified pMT-anti-LGR5(scFv)-ETA(A). Lane 1 contains protein ladder and lanes 2-8 contains eluted fractions from IMAC purification. **IMAC fractions of purified pMT-anti-CD90(scFv)-ETA(B)**. Lane 1 contains the protein ladder and lanes 2-8 contains the eluted fractions from IMAC purification. **IMAC fractions of purified pMT-anti-15EpCAM(scFv)-ETA(C)**. Lane 1 contains the protein ladder and lanes 2-8 contains the eluted fractions from IMAC purification. The protein purification was successful as the SDS PAGE analysis indicates the presence of the proteins of interest at 72kDa. The fractions were pooled and concentrated using 50kDa Amicon® Ultra-15 centrifugal filter (Sigma, USA, UFC901008). The concentrated fractions were used for SEC. The SEC fractions were concentrated and analyzed on a 10% SDS PAGE and Western blot.



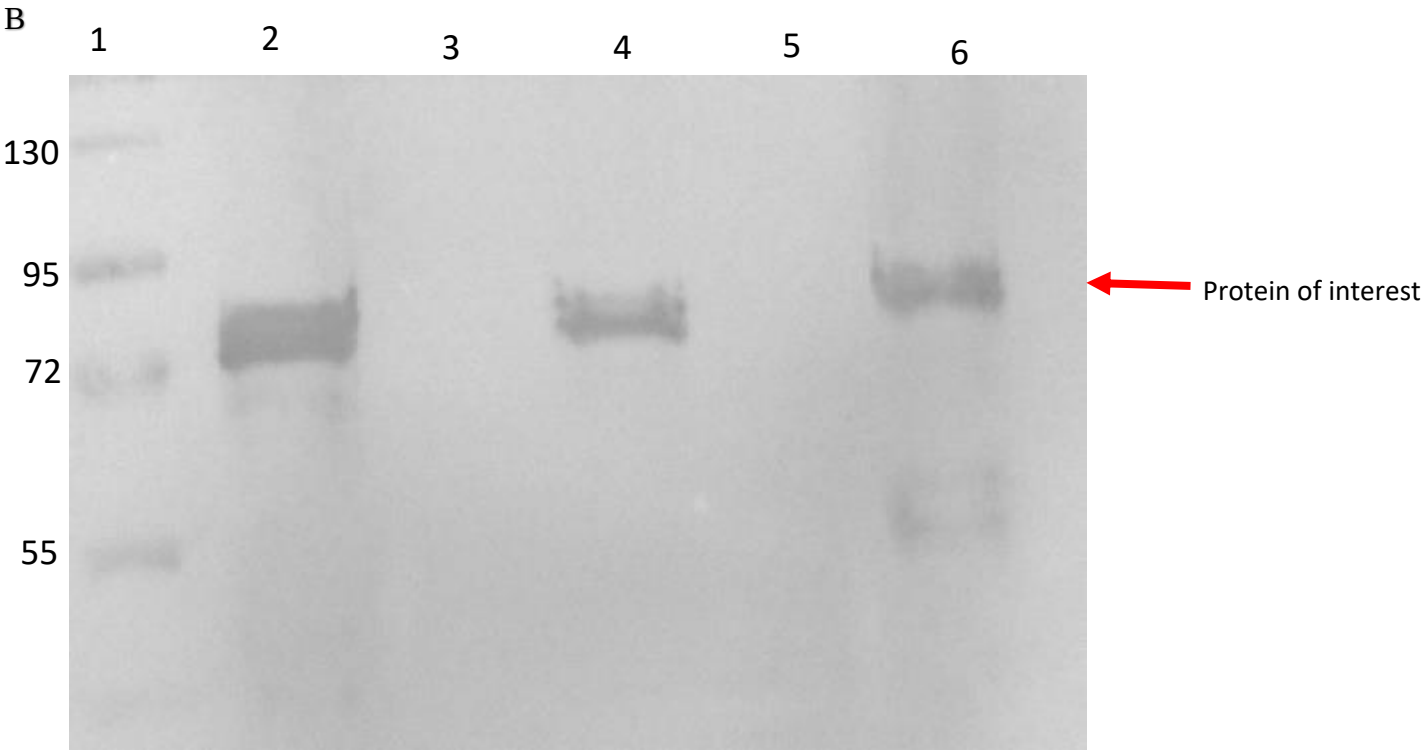


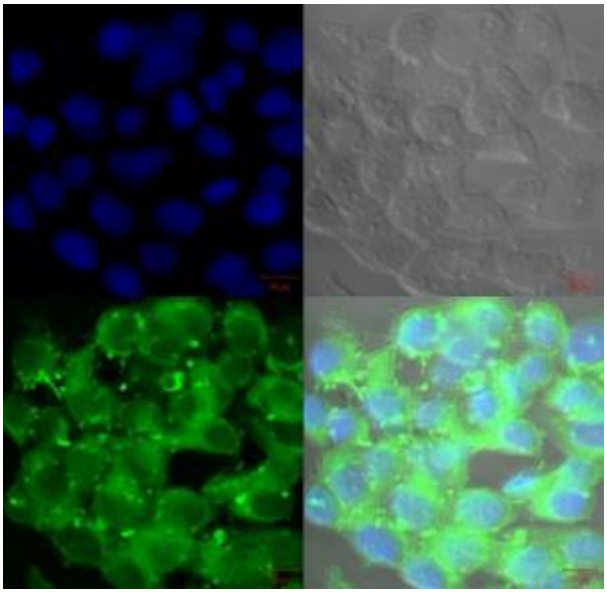
Figure 15: **SDS PAGE of purified ETA-based rITs (A)**. Lane 1 contains the protein ladder, lane 2 contains pMT-anti-LGR5(scFv)-ETA, lane 4 contains pMT-anti-CD90(scFv)-ETA and lane 6 contains pMT-anti-15EpCAM(scFv)-ETA. **Western Blot of purified ETA-based rITs (B)**. Lane 1 contains the protein ladder, lane 2 contains pMT-anti-LGR5(scFv)-ETA, lane 4 contains pMT-anti-CD90(scFv)-ETA and pMT-anti-15EpCAM(scFv)-ETA. The SEC was successful as the protein of interest was present at 72kDa (red arrow) in the SDS PAGE and Western Blot.

The Wildtype rIT's were then used for surface binding and internalization using Confocal Microscopy and Cytotoxicity assays (XTT).

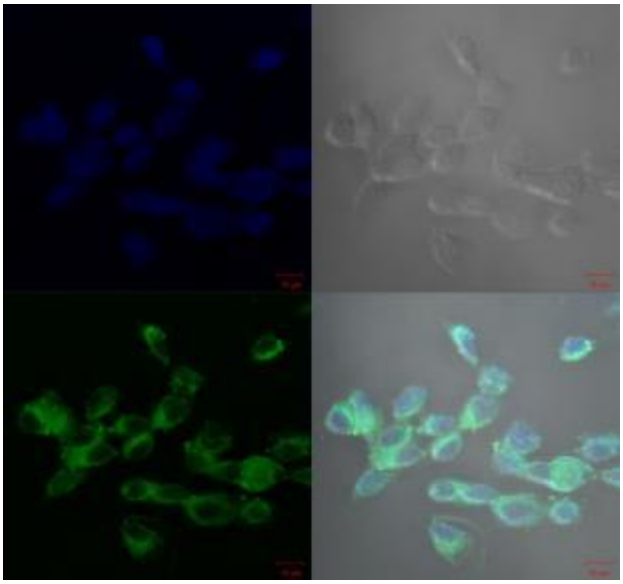
3.5) Cell Surface Binding studies

The rIT's were assessed whether they are capable of binding to the target. The sample was prepared mentioned in (2.2.7a). HL60 cell line was used as a negative antigen control for LGR5 and EpCAM rITs. MCF7 cell line was used as a negative antigen control for CD90 rITs. The antigen negative cell lines should not demonstrate cell surface binding. As a rIT negative control anti-H22(scFv)-ETA was used as no surface should occur on the CaSki cell line.

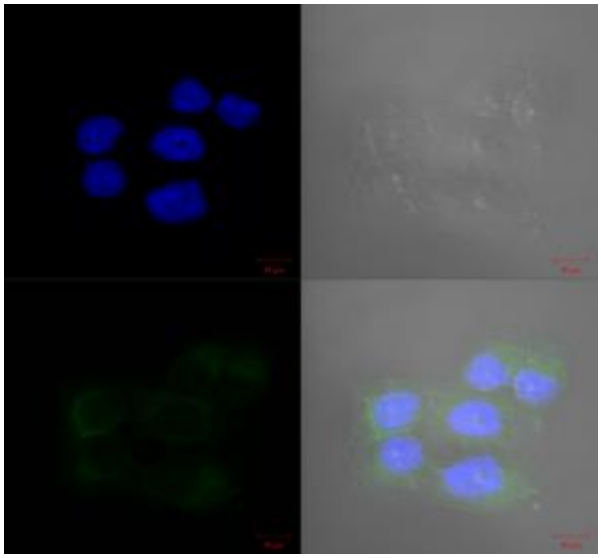
(A) anti-15EpCAM(scFv)-ETA and CaSki



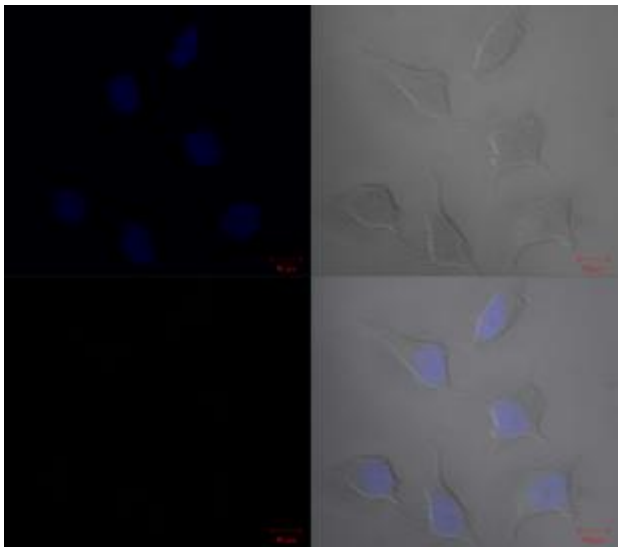
(B)anti-CD90(scFv)-ETA and CaSki



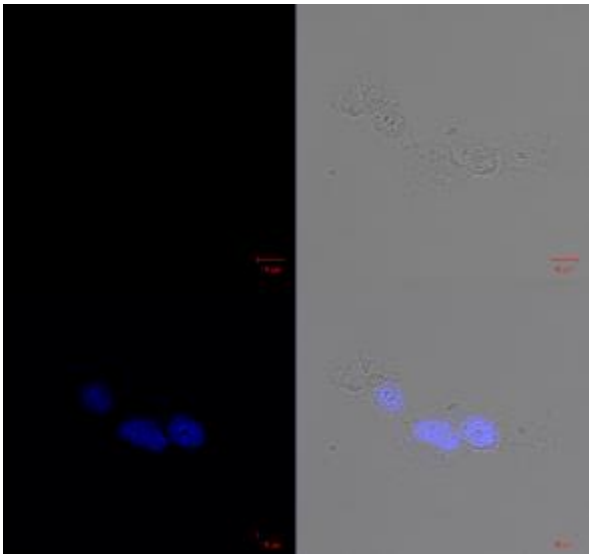
(C)anti- LGR5(scFv)-ETA and CaSKi



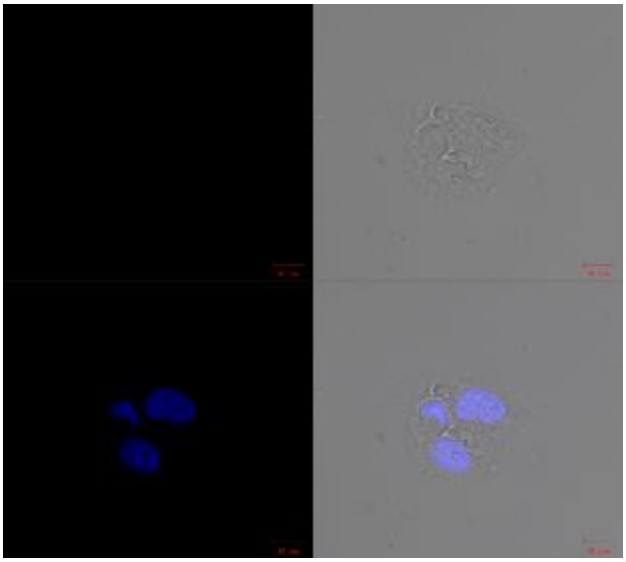
(D) anti-H22(scFv)-ETA and CaSki



(E) anti-15EpCAM(scFv)-ETA and HL60



(F) anti-CD90(scFv)-ETA and MCF7



(G) anti-LGR5(scFv)-ETA and HL60

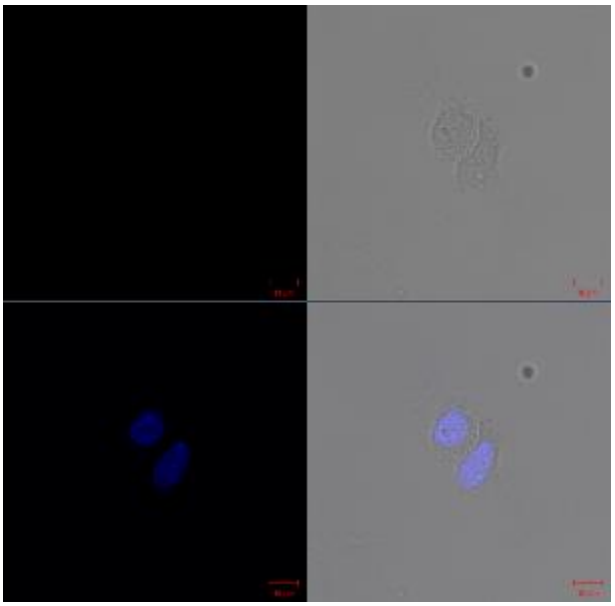
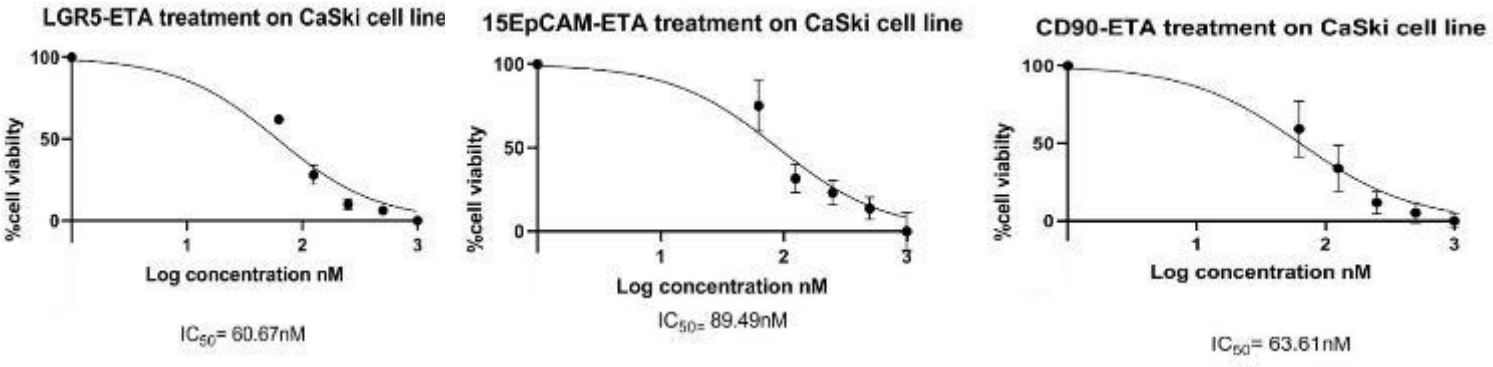


Figure 16: Confocal microscopy demonstrating surface binding on CaSki cells. Anti-15EpCAM(scFv)-ETA binding to CaSki cells (A). Anti-CD90(scFv)-ETA binding to CaSki cells (B). Anti-LGR5(scFv)-ETA binding to CaSki cells(C). Anti-H22(scFv)-ETA showing no binding to CaSki cells (D). Anti-CD90(scFv)-ETA showing no binding to MCF7 cells (E). Anti-15EpCAM(scFv)-ETA showing no binding to HL60 cells (F). Anti-LGR5(scFv)-ETA showing no binding to HL60 cells (G). Cells were incubated with Hoechst (1:5000 dilution) at 37ºC for 30 minutes to stain the nuclei(blue). The cells were then incubated with the rITs for 30 minutes at 37ºC. The cells were then incubated with anti-His-PE antibody at 37ºC for 30 minutes. Cells were washed 3 times with 1x PBS, followed by being incubated with 4% PFA at room temperature for 20 minutes to fix the cells to the coverslip. The coverslips were then mounted on a microscope slide before being viewed under a Zeiss confocal scanner microscope (LSM88) at 20µm.

3.6) Cytotoxicity analysis

After confirming the functionality of each of the rITs an XTT assay was performed to evaluate the rITs ability to kill the target cells. CaSki cells were treated with increasing concentrations of rITs(2.2.7b) and the IC_{50} value of each rITs were calculated.



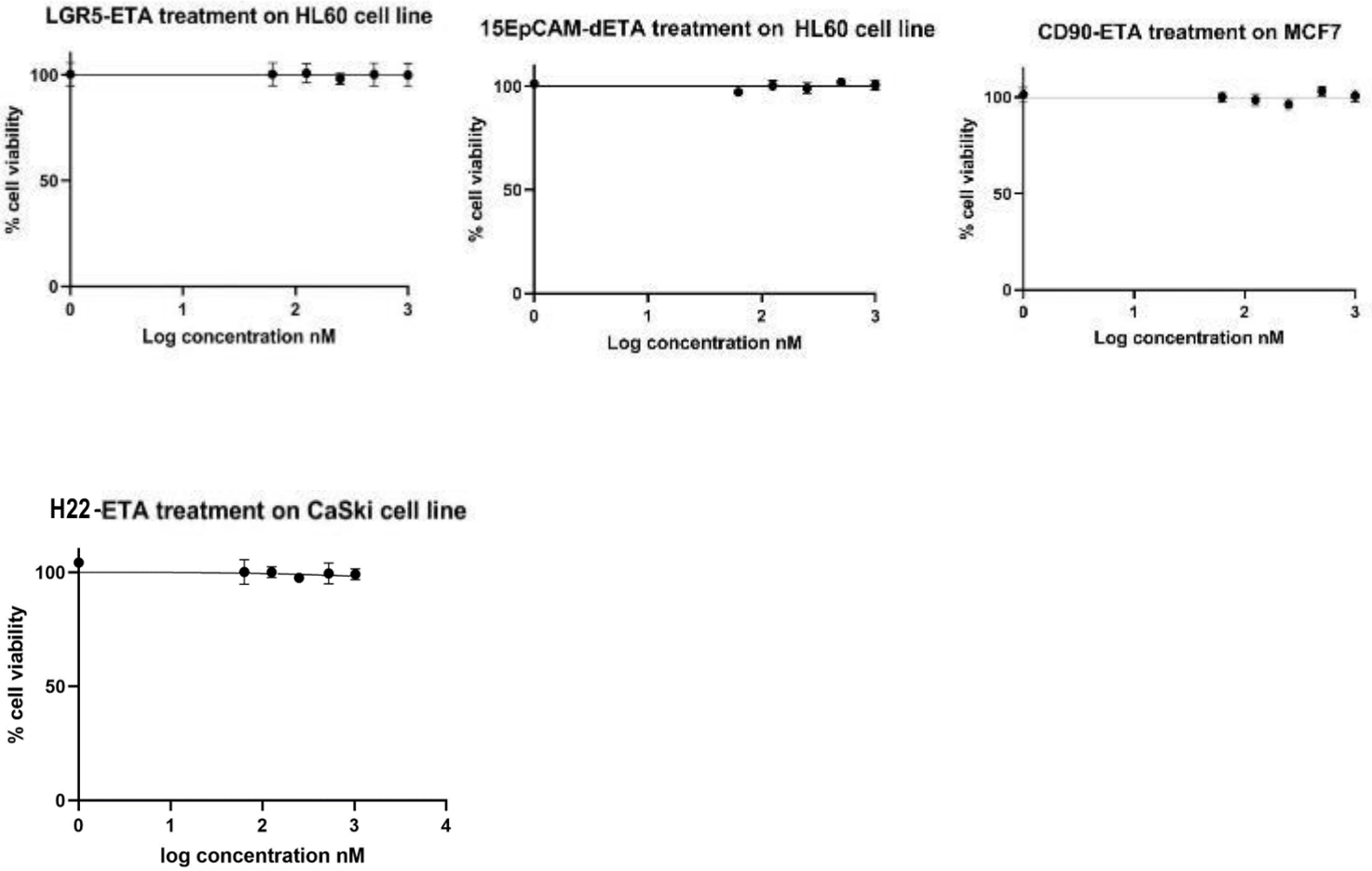


Figure 17: Cytotoxic evaluation of ETA-based rITs. The cytotoxicity activity was assessed using an XTT cell viability assay. The cells treated with serially diluted concentrations of rITs followed by performing a XTT cell viability assay. Controls were untreated (negative control) and cells treated with zeocin(100µg/ml) (positive control). The HL60 cell lines served as an antigen negative control for LGR5 and 15EpCAM rITs. The MCF7 cell line served as an antigen negative control for the CD90 rIT. Anti-H22(scFv)-ETA served as a rIT negative control for the CaSki cell line.

Table 12: Cell line dose response against ETA-based rITs

rIT	**Cell line**	**Antigen expressed**			**IC_{50}**
		LGR5	EpCAM	CD90	
Anti-LGR5(scFv)-ETA	CaSki	+	+	+	60.67nM
	HL60	-	-	+	N/A

Anti-15EpCAM(scFv)-ETA	CaSki	+	+	+	89.49nM
	HL60	-	-	+	N/A
Anti-CD90(scFv)-ETA	CaSki	+	+	+	63.61nM
	MCF7	+	+	-	N/A
Anti-H22(scFv)-ETA	CaSki	+	+	+	N/A

All ETA-based rITs demonstrated a dose response against the CaSki cell line. This coincides with the strong binding previously demonstrated in the confocal analysis. Anti-LGR5(scFv)-ETA demonstrated the most potent dose response among all three rITs. This indicates that the LGR5 receptor is not only overexpressed but also easily assessable for the rIT. The same can be claimed for anti-CD90(scFv)-ETA as it demonstrated the second most potent dose response towards the CaSki cell line. Anit-15EpCAM(scFv)-ETA had the least potent dose response towards the CaSki cell line, this may indicate that the EpCAM receptor on the CaSki cell surface may be less accessible for the rIT in comparison to the other receptors. This is observed by the difference in their IC_{50}. Anti-LGR5(scFv)-ETA and anti-CD90(scFv)-ETA being 60.67nM and 63.61nM respectively whereas the anti-15EPCAM (scFv)-ETA rIT had an IC_{50} value of 89.49nM. For the antigen negative controls, HL60 cell line expresses the CD90 biomarker but does not express the EpCAM and LGR5 biomarker. The cell viability of the HL60 cells were not affected by the anti-15EpCAM(scFv)-ETA and anti-LGR5(scFv)-ETA rITs, indicating that the rITs do not kill nonspecifically. The same can be observed with CD90-ETA on the MCF7 cell line. As 15EpCAM and LGR5 are expressed but not CD90. The cell viability of the MCF7 cells were not affected by CD90-ETA. For the negative rIT control the cell viability of the CaSki cell line was not affected by anti-H22(scFv)-ETA.

3.7) Generation of dETA-based rITs

Having proved that functional ETA-based rITs are able to be produced in the periplasmic space of *E. coli*. The next set of results focuses on the generation and production of dETA-based rITs.

The pMT-anti-H22(scFv)-ETA plasmid was used to generate the dETA-based rITs. The ETA gene was flanked by *NotI* and *BlpI*. These sites were inserted in a pUC vector containing dETA. Both plasmids were digested with the same set of restriction enzymes. The digest products were analysed on a 1.2% agarose gel.

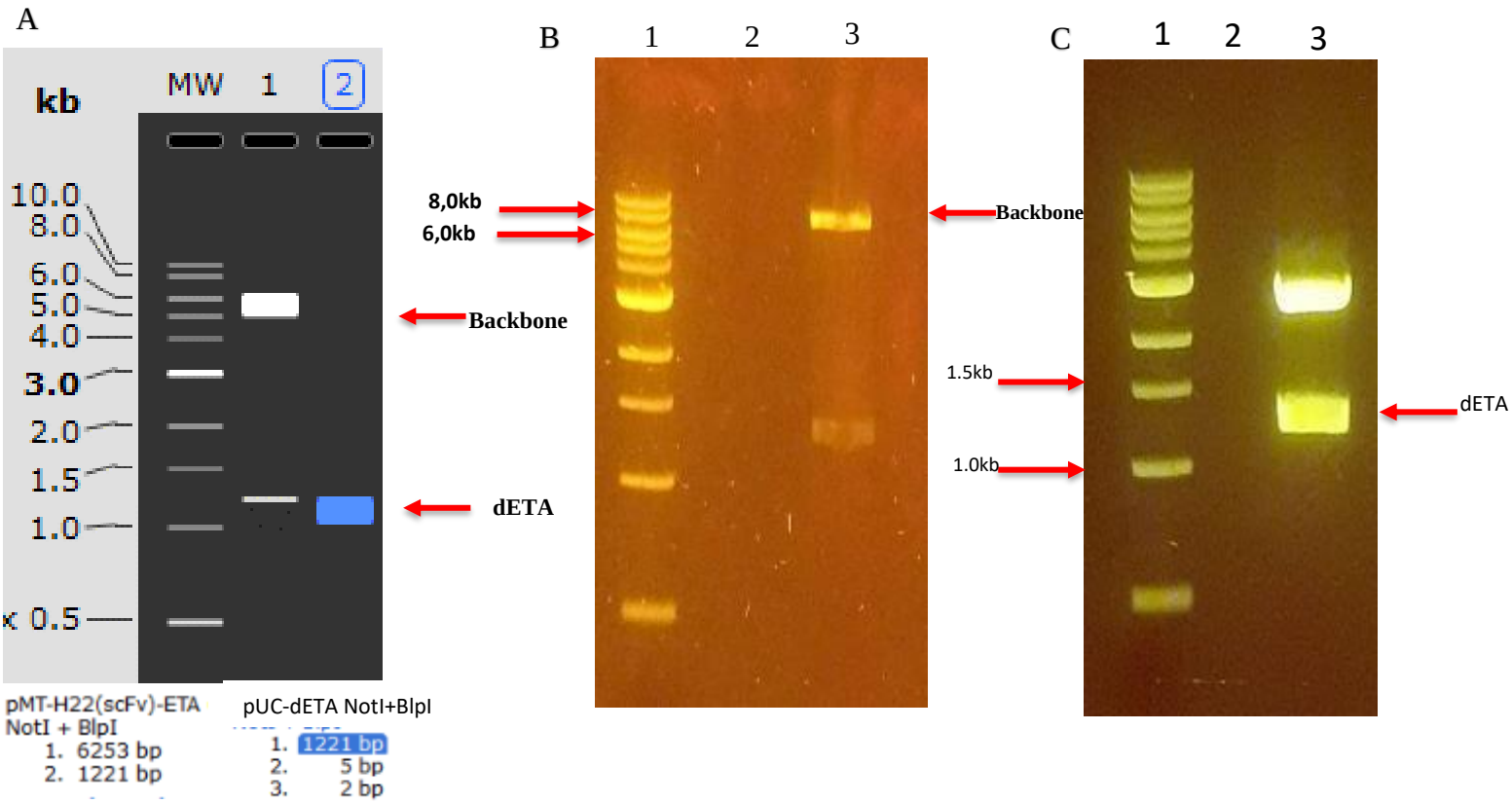


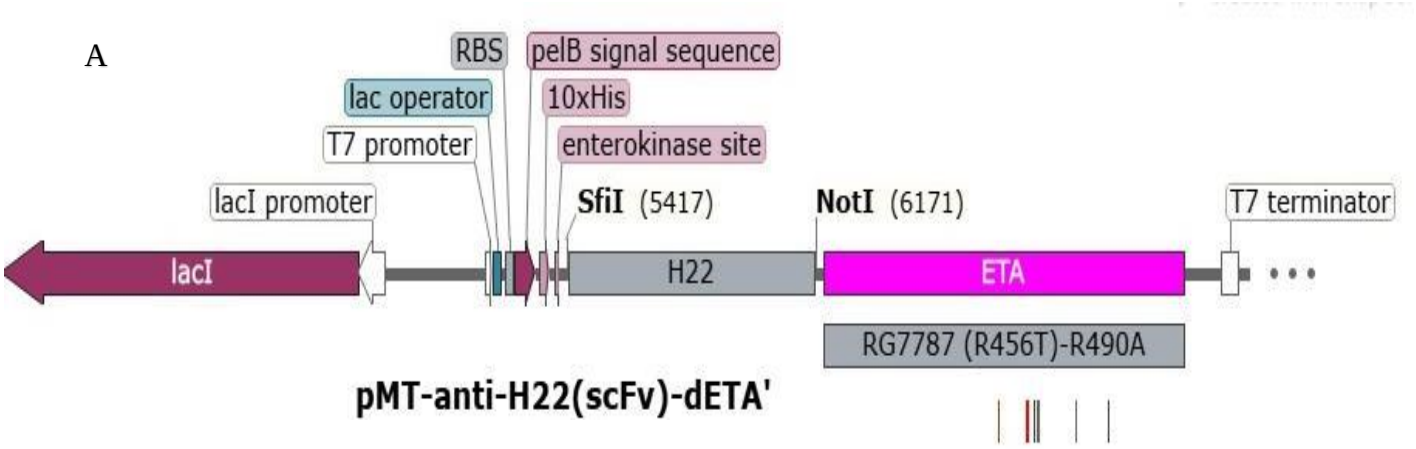
Figure 18: Simulation of the double digest of pMT-anti-H22(scFv)-ETA and pUC57-dETA(A). Mw represents the simulated marker. Lane 1 represents the simulated double digestion product of pMT-anti-H22(scFv)-ETA and Lane 2 represents the expected size of the dETA gene. Double digested product of pUC57-dETA. Lane 1 contains a 1Kb marker, lane 2 is blank and lane 3 contains the *NotI* and *BlpI* double digest product. **Double digestion product of pMT-anti-H22(scFv)-ETA(B)**. Lane 1 contains a 1Kb marker, lane 2 is blank and lane 3 contains the *NotI* and *BlpI* double digest product. **Double digestion product of pUC57-dETA (C)**. Lane 1 contains a 1Kb marker, lane 2 is blank and lane 3 contains the double digest product of *NotI* and *BlpI*.

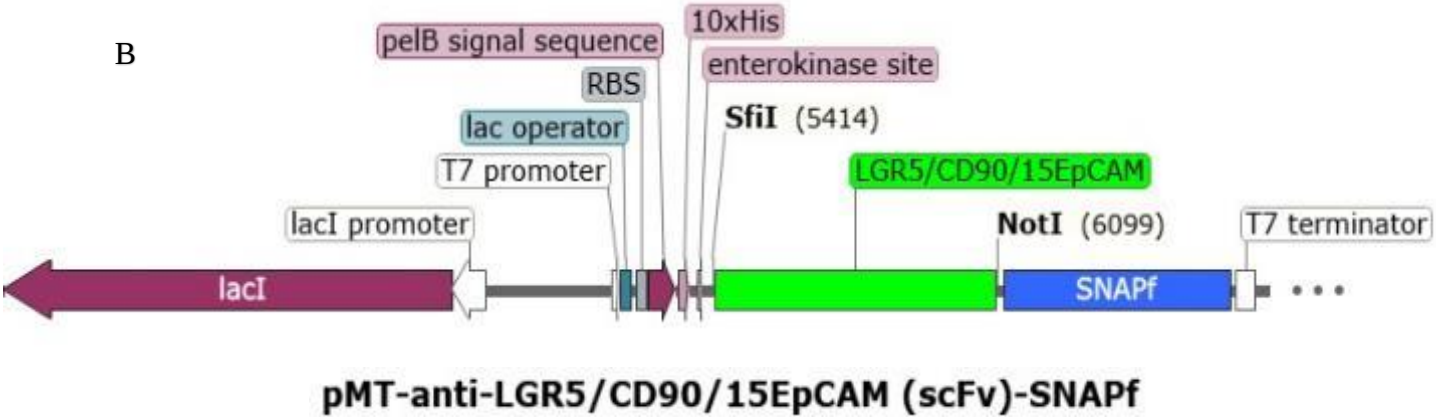
The digestion of both pMT-anti-H22(scFv)-ETA and pUC57-dETA were successful. Both digestion products exhibited a band size that corresponds with the simulation, with pMT-anti-H22(scFv)-ETA producing two bands at 6253bp and 1221bp. The band at 6253bp represented the anti-H22(scFv) containing backbone and was excised from the gel. The

dETA band corresponded with the simulation by producing a band at 1221bp. The band was excised from the agarose gel. The two bands of interest were ligated and transformed into DH5alpha *E. coli* and plated onto supplemented agar plates. Colonies were screened for positive clones via restriction mapping using *PvuII* and Sanger sequencing. Once clones were confirmed the In-silico plasmids were used for the *in-silico* cloning of the target scFvs.

3.8) *In silico* cloning of dETA-based rIT's

As the backbone came from pMT-anti-H22(scFv)-ETA plasmid, the H22 (scFv) had the *SfiI* and *NotI* restriction sites flanking it. Therefore, like the wildtype simulation, the dETA-based plasmids were used to simulate the molecular cloning procedure with pMT-SNAP plasmids containing the target scFvs.





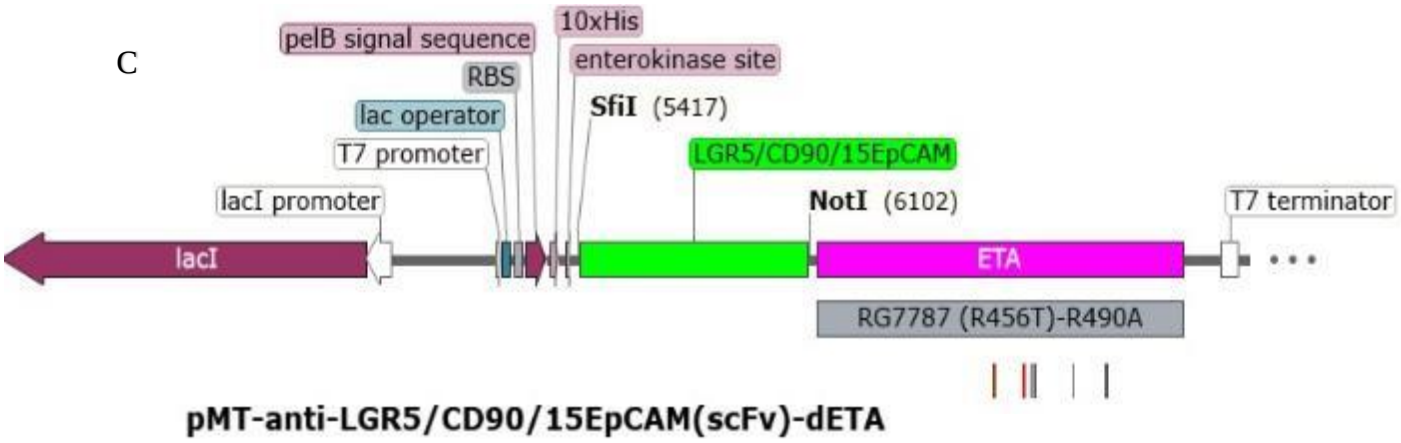


Figure 19: Schematic of the *in-silico* design of pMT-anti-H22(scFv)-dETA(A). Schematic of the *in-silico* design of pMT-anti-LGR5/CD90/15EpCAM(scFv)-SNAPf(B). Schematic of the *in-silico* design of pMT-anti-LGR5/CD90/15EpCAM(scFv)-dETA(C). The ORF of the pMT-vector for periplasmic expression contains a pelB sequence for the transport of the recombinant protein to the periplasmic space. A poly (10x) His tag for downstream processes. An enterokinase cleavage site (EKS) for post purification tag removal and the C-terminus the dETA toxin domain contains point mutations at the following positions: R427A, R456T, D463A, R467A, R505A and R538A.

Like the Wildtype variant, LGR5, CD90 and 15EpCAM replaced H22(scFv) in the dETA containing plasmid. The molecular cloning was performed in a similar manner to the Wildtype rITs. The bands of interest were identical to the Wildtype. This is because the effect of the mutations is negligible therefore the band sizes will remain the same.

The bands of interest were excised from the agarose gel and ligated and transformed into DH5alpha *E. coli* cells. The cells were plated on a supplemented agar and colonies were screened using restriction mapping and Sanger sequencing. Positive clones were isolated and transformed into BL21 *E. coli* and used for protein expression.

3.9) Protein expression and purification

BL21 *E. coli* containing dETA was cultured and produced the rITs in the periplasmic space under osmotic stress conditions. The cells were harvested and prepared the same way as the Wildtype rITs. The supernatant was purified using IMAC. The fractions were run on 10% SDS PAGE.

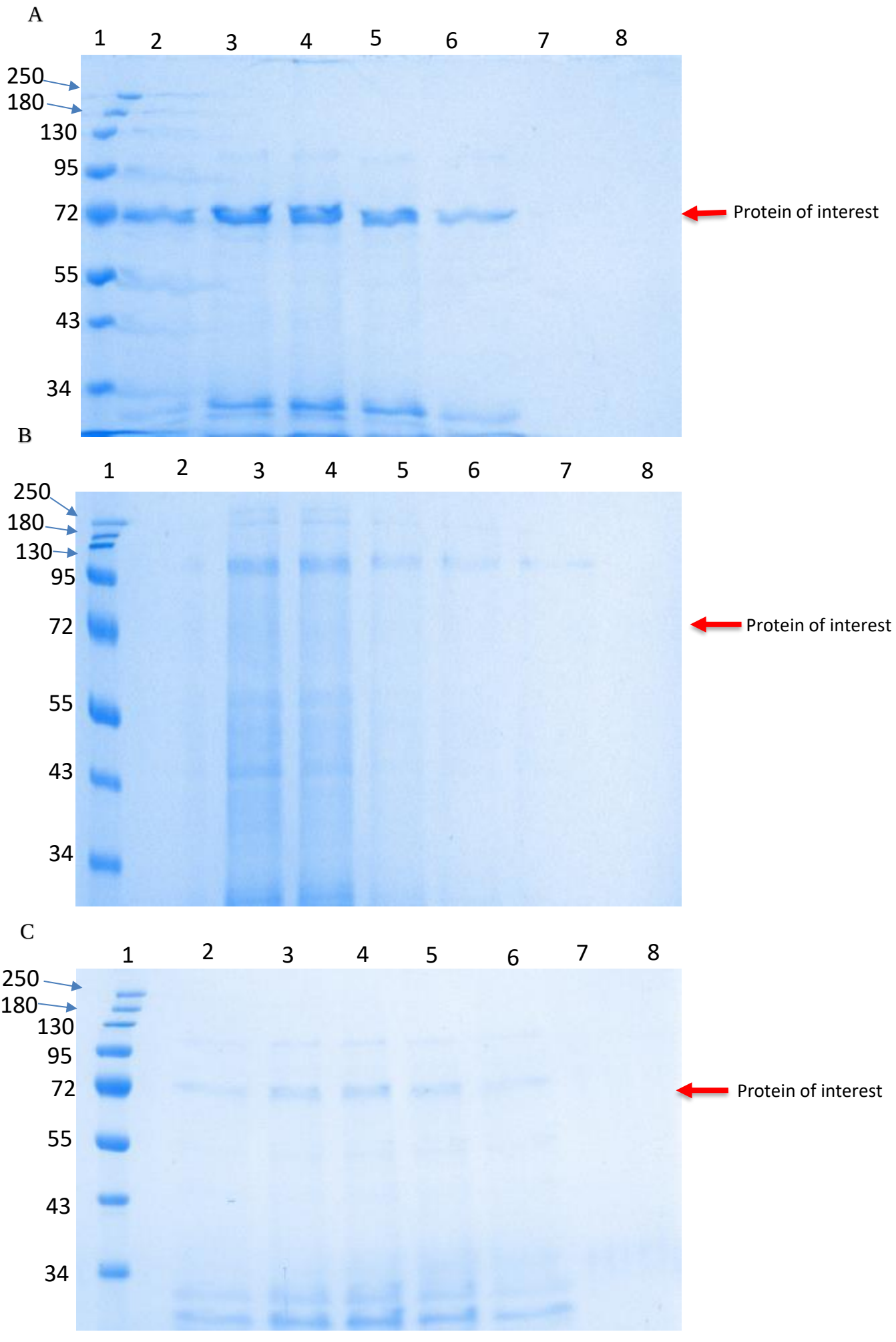

A
1 2 3 4 5 6 7 8
250
180
130
95
72
55
43
34
Protein of interest
B
1 2 3 4 5 6 7 8
250
180
130
95
72
55
43
34
Protein of interest
C
1 2 3 4 5 6 7 8
250
180
130
95
72
55
43
34
Protein of interest

Figure 20: SDS PAGE analysis of IMAC fractions of purified dETA rIT's. IMAC fractions of purified pMT-anti-LGR5(scFv)-dETA (A). Lane 1 contains the protein ladder and lanes 2-8 contains the eluted fractions from IMAC purification. **IMAC fractions of purified pMT-anti-CD90(scFv)-dETA (B).** Lane 1 contains the protein ladder and lanes 2-8 contains the eluted fractions from IMAC purification. **IMAC fractions of purified pMT-anti-15EpCAM(scFv)-dETA (C).** Lane 1 contains the protein ladder and lanes 2-8 contains the eluted fractions from IMAC purifications. The protein purification was successful as the SDS PAGE analysis indicates the presence of the proteins of interest at 72kDa.The "cleanest" fractions were selected and concentrated using 50kDa Amicon® Ultra-15 centrifugal filter (Sigma, USA, UFC901008). The concentrated fractions were used for SEC. The SEC fractions were concentrated and analyzed on a 10% SDS PAGE and Western blot.

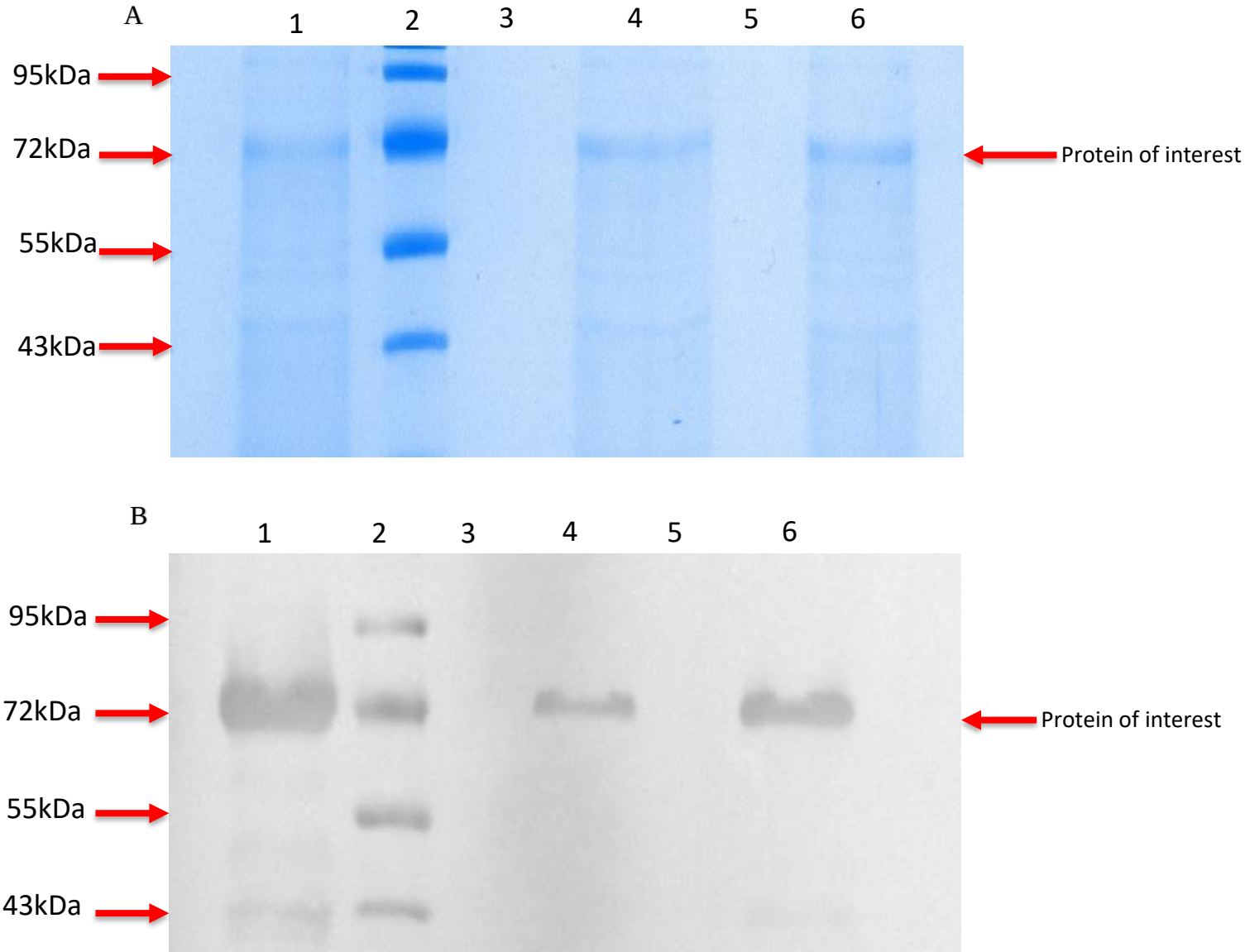
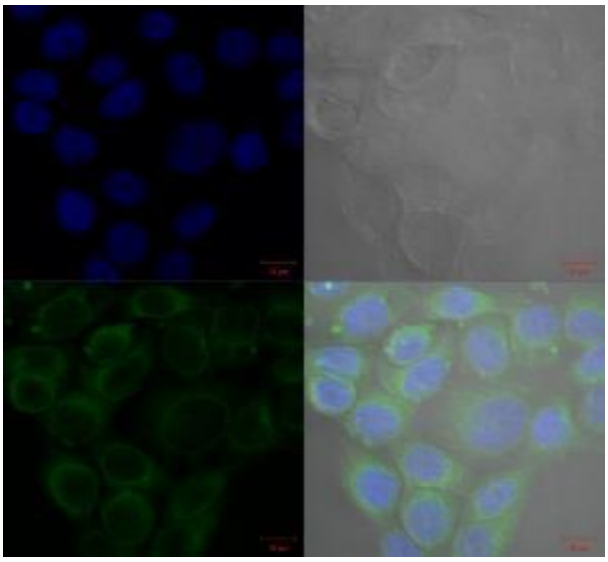


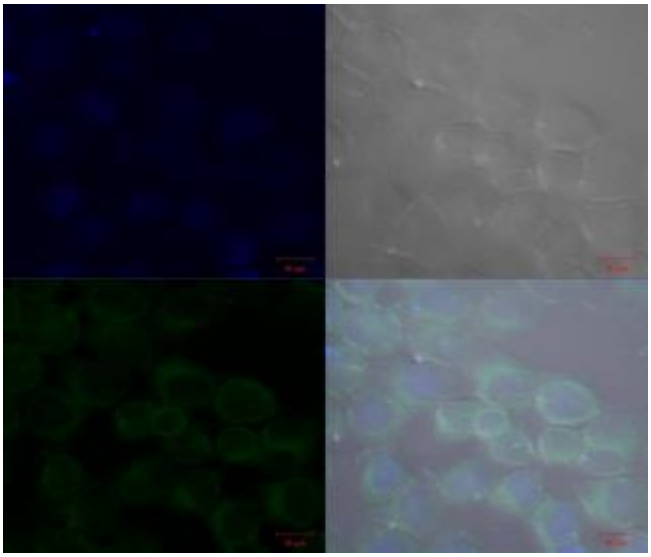
Figure 21: SDS PAGE of dETA rITs post IMAC and SEC(A). Western blots of dETA rITs Post IMAC and SEC(B). Lane 1 contains anti-LGR5-dETA, lane 2 contains the protein ladder, lanes 3 and 5 are blank, lane 4 contains anti-CD90(scFv)-dETA and lane 6 contains anti-15EpCAM(scFv)-dETA.

The dETA-based rITs were successfully purified using the combination of IMAC and SEC. This was indicated by both the SDS PAGE and Western blot. The SDS PAGE indicated the presence of a 72kDa protein as the band corresponded with the 72kDa marker on the protein ladder. The western blot indicates the protein at 72kDa has a His-tag, this confirms the presence of the dETA-based rIT. These purified rITs were used for binding studies using confocal microscopy and cytotoxicity assays using XTT cell viability assays.

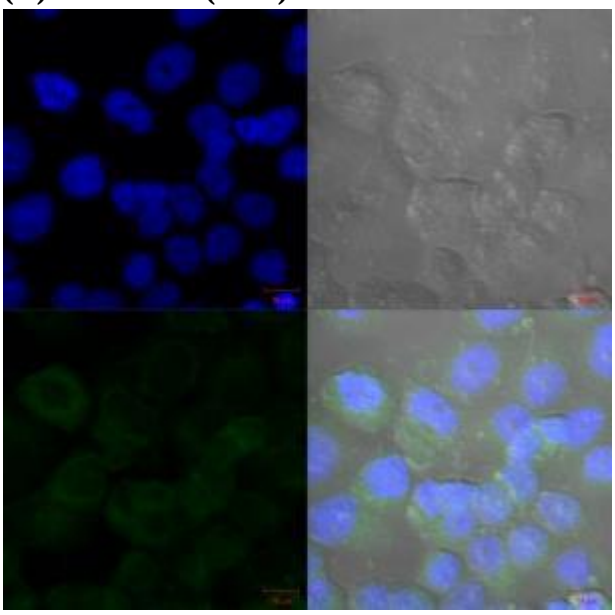
(A) anti-15EpCAM(scFv)-dETA



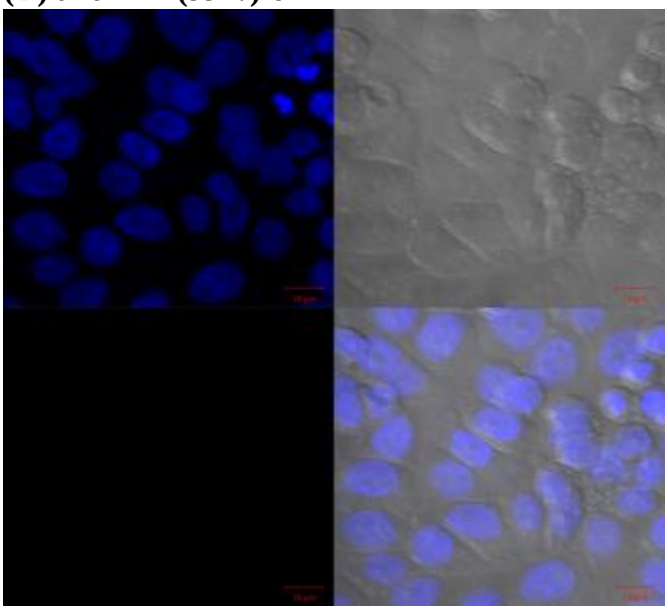
(B) anti-CD90(scFv)-dETA



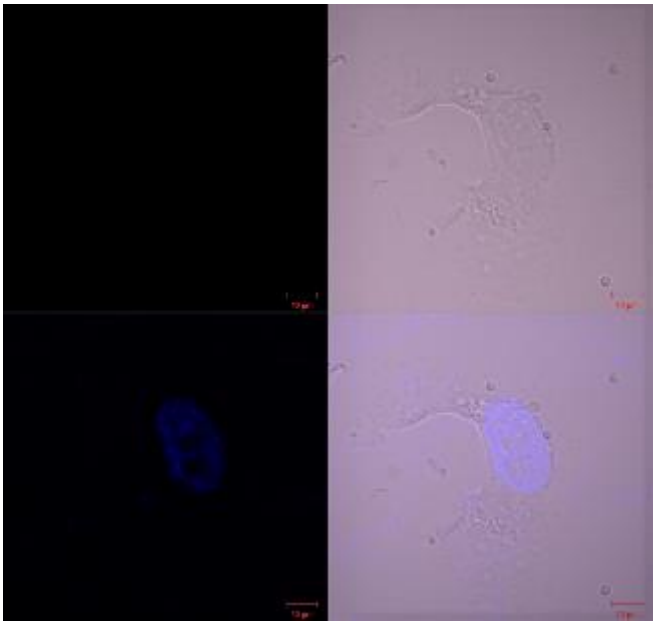
(C) anti-LGR5(scFv)-dETA



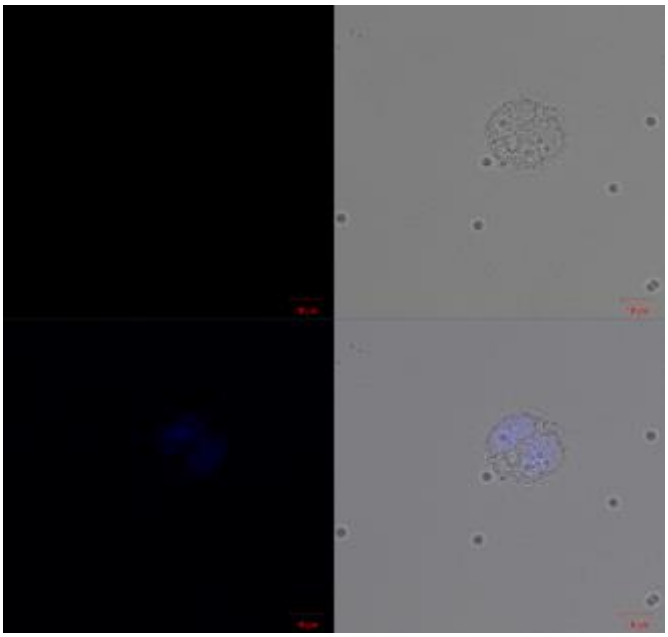
(D) anti-H22(scFv)-dETA



(E) anti-15EpCAM(scFv)-dETA on HL60



(F) anti-CD90(scFv)-dETA on MCF7



(G) anti-LGR5(scFv)-dETA on HL60

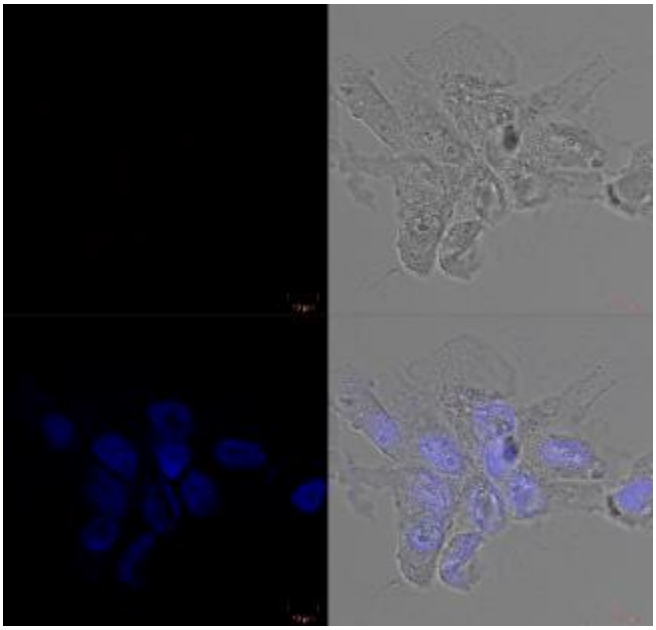


Figure 22: Confocal microscopy demonstrating surface binding on CaSki cells. Anti-15EpCAM(scFv)-dETA binding to CaSki cells (A). Anti-CD90(scFv)-dETA binding to CaSki cells (B). Anti-LGR5(scFv)-dETA binding to CaSki cells (C). Anti-H22(scFv)-dETA showing no binding to CaSki cells(D). Anti-15EpCAM(scFv)-dETA showing no binding to HL60 cells(E). Anti-CD90(scFv)-dETA showing no binding to MCF7 cells (F). Anti-LGR5(scFv)-dETA showing no binding to HL60 cells (G). Cells were incubated with Hoechst (1:5000 dilution) at 37ºC for 30 minutes to stain the nuclei (blue). The cells were then incubated with the rITs for 30 minutes at 37ºC. The cells were incubated with anti-His-PE antibody at 37ºC for 30 minutes. Cells were then washed 3 times with 1x PBS, followed by an incubation in 4% PFA at room temperature for 20 minutes to fix the cells to

the coverslip. The coverslip was then mounted on a microscope slide before being viewed under a Zeiss confocal scanner microscope (LSM880) at 20µm.

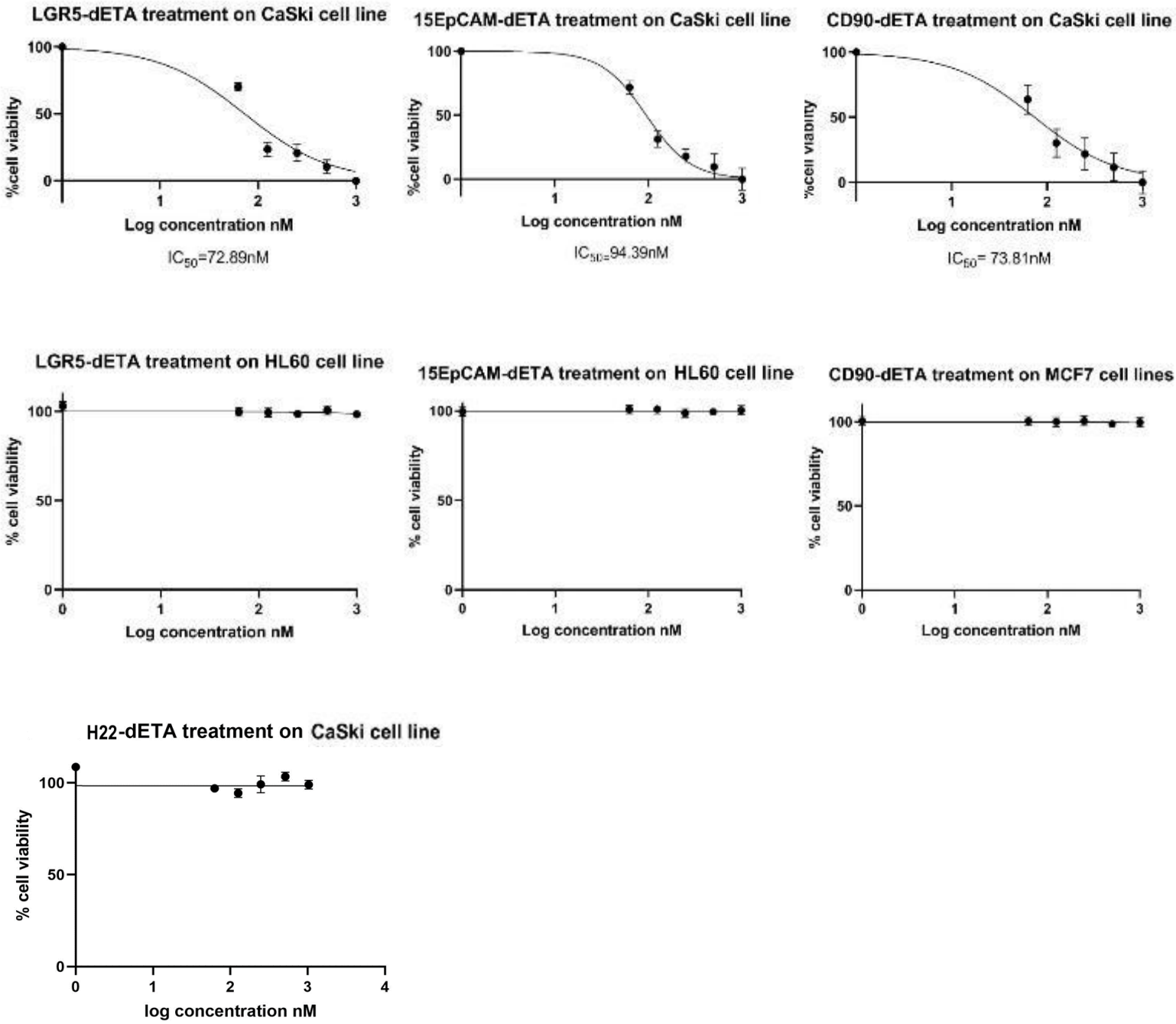


Figure 23: Cytotoxicity evaluation of dETA-based rITs. The cytotoxicity activity was assessed using an XTT cell viability assay. The cells treated with serially diluted concentrations of rITs followed by performing a XTT cell viability assay. Controls were untreated (negative control) and cells treated with zeocin(100µg/ml) (positive control). The

HL60 cell lines served as an antigen negative control for LGR5 and 15EpCAM rITs. The MCF7 cell line served as an antigen negative control for the CD90 rIT. Anti-H22(scFv)-ETA served as a rIT negative control for the CaSki cell line.

Table 13: Cell line dose response against dETA-based rITs

rIT	Cell line	Antigen expressed			IC_{50}
		LGR5	EpCAM	CD90	
Anti-LGR5-ETA	CaSki	+	+	+	72.89nM
	HL60	-	-	+	N/A
Anti-15EpCAM-ETA	CaSki	+	+	+	94.39nM
	HL60	-	-	+	N/A
Anti-CD90-ETA	CaSki	+	+	+	73.81nM
	MCF7	+	+	-	N/A
Anti-H22(scfv)-dETA	CaSki	+	+	+	N/A

All dETA-based rITs demonstrated a less potent dose response against the CaSki cell line compared to the ETA-based rITs. These results demonstrate that the potency of the toxin was affected by the point mutations introduced into the toxin domain. For the antigen-negative controls, the cell viability of the HL60 cells were not affected by the anti-15EpCAM(scFv)-dETA and anti-LGR5(scFv)-dETA rITs, indicating that the rITs do not kill nonspecifically. The same can be observed with the anti-CD90(scFv)-dETA as the cell viability of the MCF7 cells were not affected by CD90-dETA.

Chapter 4: Discussion

Most cases of cervical cancer are diagnosed in developing countries, mainly due to a lack of screening programs and vaccination strategies [169]. Approximately 83% of all new cervical cancer cases and 88% of all deaths occur in low-income areas [22]. Cervical cancer poses a significant burden in developing countries due to poor prognosis and diagnosis where women suffering from the disease have limited access to treatment.

4.1) Treatments

The stage and extent of the cancer will determine which treatment is best suited for the patient, in some cases it may include a combination of the following therapies. These treatments include surgery, radiation, and chemotherapy. Treatment options are quite limited, traditional chemotherapy has a very limited therapeutic effect and radiotherapy and surgery are prone to complications. Immunotherapy has shown promise as a new treatment for cervical cancer.

4.2) Immunotherapy

Immunotherapy has received recognition due to its sensitivity, specifically and self-renewal ability of the immune system [128]. Immunotherapy has an advantage over conventional treatments as it is capable of targeting surface antigens that are overexpressed in cancerous cells and not in normal cells. This has led to a diverse array of immunotherapeutic strategies to treat high risk, locally advanced and recurrent/metastatic cervical cancer [119]. In conjunction with the use of proteomics technology (mass spectrometry and protein array analysis) has deepened the identification of potential molecular signalling events and the proteomic characteristics of cervical cancer, which facilitates the exploration of new therapeutic agents to reduce drug resistance [128].

4.2.1) Immune checkpoint inhibitors (ICI)

ICI has provided promising but modest efficacy results in advanced cervical cancer patients with significant rates of primary resistance [177]. Pre-clinical and clinical data support the use of ICI combination approaches in different tumour types as a potential strategy to overcome resistance. Several studies have identified PDL-1 as a potential target for cervical cancer [113]. Studies have shown the increase in expression of PDL-1 in HPV-related

cervical carcinoma. This is caused by the E5, E6 and E7 oncoproteins. This has led to the development of Pembrolizumab, which is a highly selective, fully humanized monoclonal antibody that binds to PDL-1 and inhibits the PDL-1 pathway [113]. A clinical trial was performed with patients with advanced cases of cervical cancer. Patients were administered Pembrolizumab every 2 weeks for up to 24 months. The immunotherapy had an ORR of 12.5% as a monotherapy, with a median follows up time of 48.9 weeks as well as no side effects or deaths. In the phase 2 study, patients were treated with 200mg every 3 weeks. The patients ORR was 12.2% with a 10.2 month follow up. Some patients experienced a longer follow up (27 weeks) and had an ORR of 27%. These promising results promoted pembrolizumab to become FDA approved.

4.2.2) Antibody drug conjugates (ADC)

There are several clinical developments in advanced cervical cancer. Tisotumab vedotin (TV) targets tissue factor (TF), highly prevalent in cervical cancer of both squamous and adenocarcinoma histological subtypes [169]. Innova TV 204 was a multicentre phase II clinical trial designed to evaluate the activity of TV in patients with recurrent or metastatic cervical cancer. Patients received TV 20mg/Kg every three weeks until unacceptable toxicity or progression of the disease [169]. The study reported an overall response rate (ORR) of 24%, where seven patients achieved a complete response. The median duration of response (DoR) was 8.3 months. TV showed tolerability aligned with ADC safety characteristics. The most common all grade treatment -related side effects were alopecia, epistaxis, nausea and conjunctivitis [169]. The FDA approved TV in patients with persistent, recurrent or metastatic cervical cancer with disease progression after chemotherapy.

4.2.3) Recombinant immunotoxins

Wayne *et al* observed most patients who were treated with immunotoxins for solid tumours developed anti-toxin antibodies after 1 or 2 cycles of immunotoxin treatment. For immunotoxins to be effective multiple cycles of treatment needs to be administered especially in patients who develop an immune response, therefore the immunogenicity needs to be addressed [171]. In this study I addressed this issue by introducing mutations at various positions in the ETA domain. Unfortunately, the immunogenicity of these de-immunized immunotoxins could not be assessed and compared to the wildtype immunotoxins.

4.3) Protein expression in the periplasmic space

The bacterial expression system has been the preferred choice for recombinant protein production, however there are many factors that need to be considered when using *E. coli* as the expression host. The major advantage of the bacterial expression system is its cost effectiveness. The usage of *E. coli* for recombinant protein production stems from the experience of decades of research in genetics, easy manipulation, and genetic engineering tools readily available to modify the organism [73]. For instance, in this study the *E. coli* strain used for the rITs production was *E. coli BL21(DE3)* strain. This strain has defects in the genes encoding for Lon protease and OmpT protease. The system consists of the promotor lac region preceding the operon and the LacI repressor encoded by the gene [144]. The lac operon is activated by the addition of IPTG, it binds to active LacI repressor and causes the dissociation from its operator. This strain also contains a DE3 lysogen which contains T7 RNA polymerase controlled by a LacUV 5 promoter [63]. By using the signal sequence PelB, the recombinant protein can be directed to the periplasm of *E.Coli* [39]. Barth *et al* (2000) established the strategy to produce rITs in the periplasmic *E. coli* under osmotic stress. This was confirmed to be possible by expressing anti-H22(scFv)-ETA as seen in the results section (3.1). This takes advantage of *E. coli's* ability to adapt to osmotic pressure by accumulating organic compounds known as osmolytes [114]. Osmolytes such as betaine and sorbitol improve the protein solubility and protein stability by promoting protein refolding and disrupting protein aggregation [28 ;114; 121 and 130]. Betaine plays important role in regulating the free energy of the denatured state and changing the balance to improve the native state of the recombinant protein [28]. Periplasmic protein expression is supported by Joseph *et al* (2015) as the cytoplasm is a reducing environment which prevents the disulphide bonds from forming in recombinant proteins. Whereas the periplasm is a non-reducing environment which allows proteins to form disulphide bonds, promotes proper protein folding and contains fewer proteases compared to the cytoplasm [40; 50 and 73]. Periplasmic secretion has been previously used to produce functional recombinant proteins such as antibody fragments [33 and 54]. According to Joseph *et al* (2015) varying concentrations of salts, peptone and yeast extract can increase the yield of recombinant protein. During protein production, aggregation of protein in the periplasm can be suppressed by allowing the cells to grow in media containing high concentrations of sorbitol [71]. This increase in osmotic pressure results in the stabilization of the proteins native structure. The three important factors affecting the protein expression and solubility are the post-induction temperature, the

optical density and duration of the induction. Low induction temperature benefits recombinant protein production [31]. This is due to the energy conservation response to decrease the central carbon metabolism which results in a slower growth rate. Protein expression induces a metabolic burden on the host which results in an accumulation of target proteins in insoluble aggregates (inclusion bodies) [73]. The aggregation reaction is favoured at high temperatures, due to the strong temperature dependence of hydrophobic interactions between the amino acids which result into the aggregation reaction. By decreasing the induction temperature, the aggregation of proteins can be avoided. The optical density of the expression is important as cultures with high cell densities are in some cases not ideal for protein production. At high cell densities, metabolic activity of cells declines due to several factors like nutrient concentration, acetate production, less availability of dissolved oxygen and high levels of carbon dioxide decrease the recombinant gene expression [73]. High temperature may also lead to a high probability of plasmid loss due to the higher growth rate [121]. The length of induction duration is important as extended lengths of incubation may lead to protein aggregation. Many studies have utilized 16 hours as the standard duration of induction. There are some proteins that benefit from a longer induction at low temperature [121]. This further supported by Barth *et al* (2000) who accumulated 95% functional protein as compared to standard conditions where they only managed to accumulate less than 10% of functional protein as well as Mukherejee *et al* who demonstrated that *E. coli* cells were capable of secreting 90% of a recombinant single-chain antibody fragment (scFv fused to a PelB signal sequence). In this study a high level of recombinant protein was expressed as observed when performing SDS PAGE analysis. With the results in this study, it further supports that the periplasmic space of the *E. coli* is the optimum location for recombinant protein production when selecting the bacterial expression system. Unfortunately, the production chain is hampered by its poor purification ability.

4.4) Protein purification

The ideal purification should consist of a balance among several parameters that leads to the highest yield of target protein. These parameters include the speed of the purification, the recovery rate, the capacity and the resolution [88] In this study these parameters had to be altered as the initial purification runs yielded poor target protein. As confirmed by post purification analysis using SDS PAGE and Western blot showing a large quantity of non-specific proteins in the eluted fractions. Western blotting was performed to determine the

presence to the protein of interest, unfortunately early Western blots could not confirm the presence of the protein of interest. The method of purification used in this study needs to be taken into context. The first purification step utilized in this study was immobilized metal affinity chromatography (IMAC). IMAC is based on the interaction between a transition metal ion (Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) immobilized on a matrix and a specific amino acid side chain [20]. These specific amino acids can be joined to the target protein at the DNA level leading to the expression of what is known as a "tag" on the target protein. There are several affinity tags available such as Histidine tag (His-tag), Arg-tag, glutathione S-transferase (GST) tag and Strep tag II [88]. For this study a His-tag was used for the purification of the target protein. The reason for this choice of affinity tag is due to its advantages over the other affinity tags. His-tag possess a plethora of advantages over the other affinity tags, firstly the tag can easily be added to the protein of interest. The poly-His-tag is usually placed on either the N or C terminus of the target protein during the genetic engineering step. His-tags has been more commonly used due to their small size and are less disruptive to the function of the target protein compared to other affinity tags [88]. Histidine is the amino acid that exhibits the strongest interaction with the immobilized metal metrices as the electron donor groups on the histidine imidazole ring readily from coordination binds with the immobilized transition metal [21]. In tandem with that property, a poly-His-tag can be eluted under mild conditions from the IMAC resin, which allows the target protein to retain its biological function. Although His-tag affinity tag hold many advantages over other affinity tags it has some disadvantages namely: the nonspecific binding of proteins onto the IMAC column. In some cases, the use of other affinity tag systems resulted in higher degrees of enrichment of the affinity-tagged protein than can be attained when using a poly-His-tag affinity system [21]. A good example of this is the use of biotinylating- accepting domain affinity tag have shown to provide a higher yield of target protein compared to the poly-His-tag affinity tags. This was a major hurdle during this study, however there were parameters that were altered to achieve the best yield possible. Impurities with similar properties to the target protein hamper the yield of the purified target protein during the purification process [88]. According to Bornhorst and Falke (2000) this is a major issue when using the poly-His-tag affinity tag for purification as some cellular proteins contain 2 or more adjacent histidine residues, which have an affinity to the IMAC matrix and may coelute with the target protein resulting in contamination of the final product. Studies suggest that the use of 10mM 2-mercaptoethanol in the loading, washing or elution buffer would possibly eliminate these issues. They also suggest that nonspecific hydrophobic interactions with the IMAC matrix may be a possible

reason for coelution of nonspecific protein. To combat this, low levels of non-ionic detergent such as Triton X-100 or Tween 20 in the buffers may reduce the interactions of nonspecific proteins without affecting the binding of the target protein with the IMAC matrix. In addition, the addition of salt (500mM NaCl), glycerol (20%) or low levels of ethanol (20%) can also reduce the amount of nonspecific hydrophobic proteins from interacting to the IMAC matrix [21]. Low levels of imidazole (20mM up to 50mM) may also be included in the binding or wash buffer to reduce the amount of nonspecific protein coeluted with the target protein. In this study the non-ionic detergent Triton X-100 was attempted but to no avail. The combination that yielded the best purity was a binding buffer consisting of 300mM NaCl, 10% Glycerol ,100mM Tris-HCl and 150mM Imidazole and a wash buffer consisting of 20mM $NaPO_3$, 500mM NaCl and 40mM imidazole. The imidazole concentration in the binding buffer is much higher than what other studies recommend due to the high level of contaminating proteins experienced. When the imidazole concentration would exceed 150mM the target protein wouldn't bind to the IMAC matrix. Therefore, the concentration of 150mM imidazole in the binding buffer reduced a significant amount of nonspecific protein during the purification.

The metal used for IMAC is just as important as various metals possess their own binding capacity with His-tag proteins. In principle the target protein can be captured from the cell lysate via the specific binding of the poly-His-tag to the metal chelates in the IMAC matrix. IMAC resin however have a relatively low binding capacity compared to other chromatography resins and variability in resin selectively results in additional chromatography steps to complete purification [133]. Studies have found that chromatography performed with large diameter IMAC agarose beads allowed for a higher yield of target protein. For the purifications done in this study the 5ml IMAC column was used. A poor binding capability may also be caused by certain reagents in the binding buffer. As mentioned earlier , during the previous purifications detergents such as Triton-X may have negatively affected the interaction between the protein and resin. This allowed for the most surface area for the target protein to bind to the matrix. Riguero *et al* observed that charging the resin with Ni^{2+}, Co^{2+}, Zn^{2+} or Cu^{2+} resulted in a higher binding capacity at 10% breakthrough compared to pre-charged Ni^{2+} resin. They observed that the resins charged with $Mg^{2+,}$ Mn^{2+} and Fe^{2+} exhibited a lower binding capacity compared to resins charged with Ni^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+}. Riguero *et al* examined the breakthrough profiles on IMAC 6 Sepharose columns (fig 24).

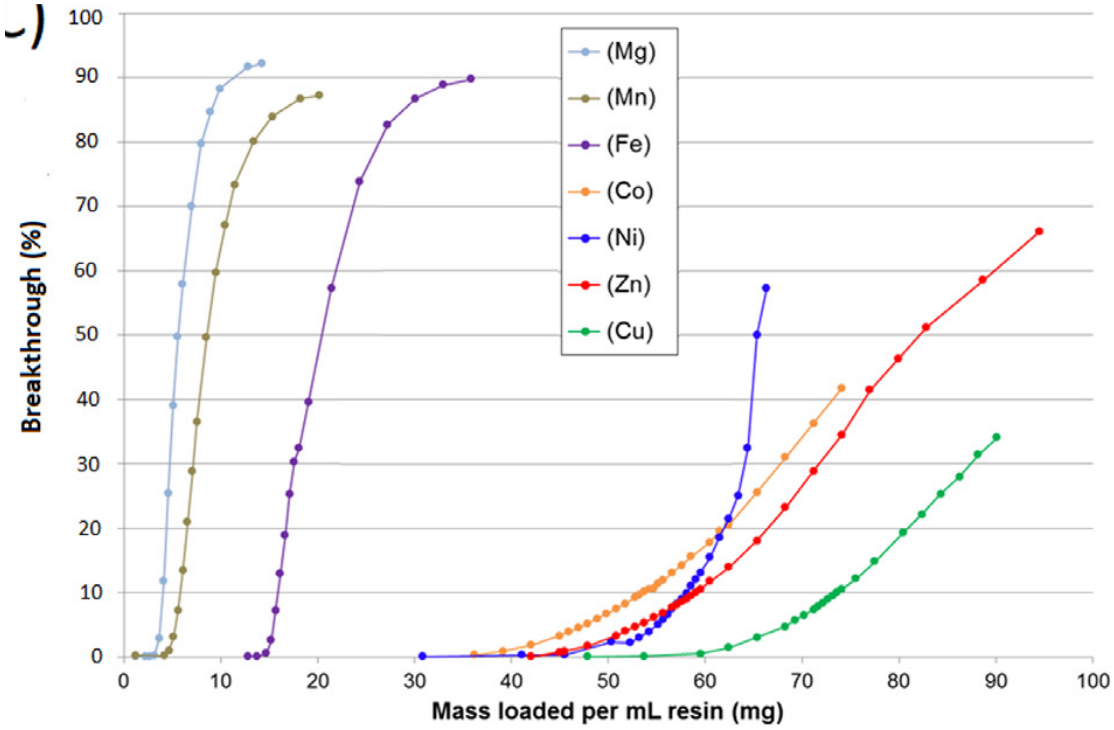


Figure 24: Dynamic binding capacities of purified protein [21]. The examination revealed that charging the resins with Co^{2+}, Zn^{2+} and Cu^{2+} resulted in a better binding indicated by the breakthrough curves generated with the other metals showed steep slopes or lower capabilities at 10% breakthrough. The study concluded that the best metal to use for IMAC is Cu^{2+} due to it having the most favourable binding profile and the highest dynamic binding capacity.

For this study the metal used for IMAC was Co^{2+} due to its weak binding to tagged proteins. This may seem counter intuitive but due to Co^{2+} weak bind capability with His-tag proteins it'll reduce the number of contaminating proteins. The target protein has 10 histidine poly His-tag which would have a higher chance of binding to the Co^{2+} resin as opposed to the other nonspecific proteins. Co^{2+} has also been used as an alternative to Ni^{2+}, since Ni^{2+} has a strong binding capability to histidine bearing proteins. On the other hand, according to literature Cu^{2+} is a resin usually used for the purification of untagged proteins and Cu^{2+} has a

strong binding capability to a wide range of proteins, this would be detrimental when the goal is to separate a specific protein from a variety of proteins.

Another potential problem when it comes to purification is the accessibility of his tag to the IMAC resin due to occlusion of the tag in the folded protein [21]. A way to solve this is to perform the purification under denaturing conditions. In this study this was not performed as there was no guarantee that the target protein would refold to its native structure as well as there was no telling what denaturing it would do to its functionality.

When performing protein purification, the physiochemical properties of the target protein and the required purification level are the most influential factors that determine the final purification strategy [88]. For this study IMAC was used as the initial purification step since it can be effectively used to purify target proteins from crude cell lysate and following IMAC purification the target protein can be further purified using other chromatography techniques i.e., Ion exchange or size exclusion. Overall, the use of IMAC to purify tagged proteins provides a rapid and inexpensive purification method in comparison to other affinity protein purification methods [21]. The IMAC purification procedure was performed in accordance with Barth *et al* (2000). Where the cell lysate should be in a buffer with a pH 8 and should be loaded slowly onto the IMAC column. This would ensure the target protein has enough time to strongly chelate on the metal within the column. The pH was slightly basic to hinder any protease activity as well as to keep the histidine residues protonated to ensure good binding. After binding, the column can be washed to remove nonspecific proteins that bind weakly to the column imidazole (10-50mM) in the wash buffer will increase the stringency of the wash and elute nonspecific bound proteins. An alternative, the column can be washed with buffer with a lower pH than the binding buffer this would remove any nonspecific protein bound to the column also the addition of agents such as 2-mercatoethanol can be included in the wash buffer to remove any nonspecific proteins. Targeted proteins can be eluted using two different methods. The first involves lowering the pH inside the column which protonates the imidazole nitrogen atom of the histidine residue which leads to the disruption of the interaction between the histidine and the metal of the column. The second involves the histidine analog imidazole, where it can be used to competitively elute the bound poly-His-tag protein. The above mentioned was used as a template to design the IMAC purification procedure. The pH of all buffers remained at pH8, and imidazole was used to wash any nonspecific proteins bound to the IMAC column and the target protein was eluted by using a

high concentration of imidazole(500mM). As a polishing step size exclusion was used to remove any remaining containing proteins.

4.5) Functionality and Cytotoxicity analysis

The cells used for this study were adherent cells therefore the surface binding of the rITs were assessed using confocal microscopy. Confocal microscopy has provided an effective means of visualizing interactions of the antibodies and their target receptors. These features associated with confocal microscopy makes it well suited to applications that require the accurate analysis of sub-cellular features in homogeneous samples, but poorly suited to detecting faint fluorescent probes or evaluating statistically significant numbers of cells within heterogeneous samples .The sensitivity of the confocal microscopy may be increased in a single image by dwelling over the cell for extended periods of time, however the this may cause photobleaching outside the plane of focus and ultimately affect the quality of the image. During this study, pilot experiment demonstrated very faint signals of cell surface binding. In order to achieve a "good" signal the incubation times with the cells and labelled probe had to be increased. The rITs also worked best when freshy purified; rITs that were purified and stored for a prolonged time failed to produce any noticeable signal during confocal microscopy analyses. This demonstrates that the rIT cannot be stored for too long as it compromises the functionality of the rIT. Therefore, the stability of these rIT in storage conditions needs to be monitored and improved for future studies.

An alternative for assessing the surface binding of the rITs is flow cytometry. Flow cytometry sacrifices imaging in favour of high acquisition rates and fluorescence sensitivity. In flow cytometry, each detection event (cell) is associated with several numerical measurements of fluorescence intensity using the forward and side scatter produced by the laser light. Forward scatter correlates to the cell size and the side scatter correlates to the cell's granularity. The major advantage of flow cytometry is that it enables one to rapidly analyse a large population of cells. Flow cytometry enables a high throughput, concurrent analysis of cell morphology and multichannel fluorescence imaging of single cells [96]. With these properties flow cytometry is a good alternative to confocal microscopy which otherwise focuses on a small portion of the cell population.

For the study CaSki cells were used to assess the binding capability of the rITs. Although the results were successful, future studies should incorporate more cell lines i.e., Me180 and

HeLa. Eventually the studies should focus on patient derived samples as the cell lines do not represent the diversity of human tumours. Established cell lines are selected from specific tumour subsets that grow under *in vitro* culture conditions [110]. This limits cell lines in the sense that there is no *in vitro* adaptation of the cells to the culture which leads to discrepancy between the experimental and clinical outcomes. There are two alternatives for the use of cell lines each having their own advantages and disadvantages. These alternatives are Patient-derived xenografts (PDX) and patient derived cancer cells. These hold the advantage over cell lines in the sense that these retain the characteristics of the original tumour including tumour heterogeneity and complexity.

PDX are grown in immunocompromised mice. This allows the PDX to maintain the key molecular abbreviations present in patient tumours, including mutations, structural genomic events and epigenetic features [110]. With these properties PDX can capture the molecular diversity, cellular heterogeneity and histology in patient tumours. PDX also helps in evaluating both the anticancer efficacy and toxicity which provides researchers an accurate therapeutic index for novel therapeutics. PDXs are an important preclinical cancer model for overcoming limitations associated with the use of cancer cell lines and allow investigators to obtain preclinical results that more accurately reflect clinical response in patient [110].

For the cytotoxicity assays cell lines are often used due to their cost effectiveness, ease of use, they provide an unlimited supply of material and bypass ethic concerns. Cell lines also provide a pure population of cells which allows for reproducible results. An XTT colorimetric assay was used to assess the cytotoxicity of the rITs. The principle of a colorimetric assay is the measurement of a biochemical marker to evaluate the metabolic activity if the cells [6]. An XTT simply measures the proliferation of the cells, it is based on the ability to reduce tetrazolium salt XTT to orange-coloured formazan compounds caused by the metabolic activity of the cells. The orange colour formazan intensity can be measured with a spectrophotometer. The IC_{50} values indicate that the CaSki cell line were more sensitive towards the ETA-based rITs compared to the dETA-based rITs. This indicates that the mutations present in the toxin domain affects the enzymatic activity of the dETA-based rITs.

Alewine *et al* (2015) demonstrated the efficacy of the RG7787. SS1(dsFv) PE38 (SS1P) is a PE38-based rIT targeted against mesothelin receptor [3]. During a phase 1 clinical trial, SS1P

demonstrated limited therapeutic effect as 90% of patients developed neutralizing antibodies against the bacterial toxin after one cycle of treatment. This led to the development of the next generation of ETA based rITs called PE24. PE24 possess similar in vitro activity as the wt PE38 but is less reactive with human anti-sera. The PE24 platform was used to develop a new mesothelin targeted rIT called RG7787 [3]. The RG7787 rIT maintains the stability and binding properties as PE24 but has the added benefit of being less immunogenic with 7 mutated B cell epitopes and a longer half-life. During the study the SS1P, its PE24-derivatives SS1-LR (wt) and RG7787(dETA) rITs were evaluated on mesothelin expressing Triple negative Breast cancer (TBNC) and Gastric cancer cell lines. For triple negative breast cancer (TNBC) cell lines HCC70 and SUM149 were used. The HCC70 demonstrated a 2 times higher sensitivity towards SS1-LR (IC_{50} 19.6 pM) compared to RG7787 (IC_{50} 45.2pM). The SUM149 cell line showed the same trend SS1-LR (IC_{50} 30.2pM) compared to RG7787 (IC_{50} 64.8pM). These results indicate that their deimmunized PE24 was less active compared to their wt PE24. These IC_{50} values are interesting as SUM149 cells overexpress mesothelin 33% more than HCC70 cells, yet the HCC70 cells demonstrate the more potent response. The results from the TNBC cell line indicate that the RG7787 rIT seems to be the superior form of treatment however, when they evaluated the rIT on the Gastric cancer cell lines three out of the four cell lines demonstrated to be more sensitive towards SS1P than RG7787. Cell lines MKN7, MKN28, MKN45 and MKN74 were used for their study. The MKN45 cell line proved to be more sensitive to the RG7787 (IC_{50} 6.8pM) compared to SS1P (IC_{50} 37.1pM), while the other cell lines (MKN7, MKN28 and MKN74) demonstrated to be more sensitive to SS1P than RG7787. SS1P achieved an IC_{50} values in the range of 5.5pM to 37.1pM, whereas RG7787 achieved IC_{50} values ranging from 75.6pM to >1200pM. This study proved that RG7787 does not always guarantee similar or improved enzymatic activity in comparison to its wt counterpart.

In consequence, during this study the point mutations R427A, R456T, D463A, R467A, R505A and R538A into the ETA domain were successfully fused to three different scFv targeting EpCAM, CD90 and LGR5. The dETA toxin and targeting scFv were successfully cloned within a pMT expression vector. These dETA-based rITs were able to be successfully expressed within the periplasm of *E. coli* BL21 and purified. These dETA-based rITs demonstrated a similar binding capacity to target cells as well as exhibiting targeted cell killing as the ETA-based rITs.

Mazor *et al* (2016) are describing several B-cell depleted variants of PE most which are demonstrating less enzymatic activity compared to wt. The same holds true for the B cell depleted RG7787 variant. The differences in the published RG7787 variants relative to wt are 2-fold less effective for the mesothelin targeting constructs and are less than 2-fold in our EpCAM, CD90 and LGR5 targeting constructs. This is not necessarily confirming the hypothesis from the computer simulation for the R456T mutant. The trend seen in this study is reproduced by another MSc student who has shown similar preliminary results for EpCAM and LGR5 targeting $dETA_{R456T}$ constructs but not for anti-CD90(scFv)-$dETA_{R456T}$ on a TNBC cell line. Which might somehow confirm an improved enzymatic activity of the R456T mutation in comparison to the original R456A.

Although the dETA-based rITs are not as potent towards the target cells, the results indicate that both ETA-based and dETA-based rIT are both capable of targeted cell killing. As previously mentioned, various cervical cell lines should be assessed to determine whether dETA-based rITs can exhibit similar or better cytotoxic activity as the ETA-based rITs. Future studies should utilise mouse models to assess the immunogenicity of both the ETA-based and dETA-based rITs.

Chapter 5: Conclusion and Future work

Overall, during the study, the various scFvs were successfully cloned within both the ETA and dETA plasmid backbones. The cloned rITs were also successfully able to be expressed within the periplasm of *E. coli* BL21. The study proved that the periplasm can be utilized in generating complex fusion proteins due to its nonreducing environment. The periplasmic expressed protein was also successfully purified using a combination of IMAC and SEC. Both ETA-based and dETA-based rITs showed a strong binding and demonstrated a potent response against CaSki cell line. Although the ETA-based rITs demonstrated a more potent response this could be due to the various point mutations within the dETA-based rITs affecting the enzymatic activity of the toxin. Future studies should implement the use of other cervical cancer lines and eventually assess the binding capability and potency against patient tissue samples. The ETA-based and dETA-based rITs should be assessed on mice models to evaluate the immunogenicity of each rITs. When cervical cancer develops the strategy should focus on preventing relapse in the patient. Therefore, the result of this thesis showcases that this form of treatment may be an alternative treatment when treating patients experiencing advanced stages of cervical cancer.

Chapter 6: References

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