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Novel Drug Delivery Platform for the Topical Treatment of Cervical Cancer

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Novel Drug Delivery Platform for the Topical Treatment of Cervical Cancer

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November 2016

Australia

Signed Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the dissertation contains no material previously published or written by another person except where due reference is made in the thesis itself.

Yaman Tayyar

November 2016

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Abstract

Background and Aims:

Cervical cancer was ranked fourth of all cancer deaths among women globally in 2012. Although vaccines were developed as prophylaxis, they do not cure existing infection, nor do they provide protection against all types of the causative virus (Human Papillomavirus). The current treatments of cervical cancer, including surgery, radiotherapy, and chemotherapy, have not improved the 5-year survival rate over the last decades, and were associated with undesirable systemic side effects. Therefore, there is a pressing need of novel strategies for cervical cancer treatment.

Aurora A Kinase was recently identified as critical for the survival of human-papillomavirus-transformed cervical cancer, which accounts for more than 99% of cervical cancer cases, and a complete regression of the disease was achieved in mouse models by inhibiting this enzyme using Alisertib (MLN8237) by Takeda (Japan). This effect was due to Alisertib sensitivity induced by the HPV oncogene, E7, providing a rationale for testing this drug to treat - HPV-driven cancers.

Alisertib is presently being assessed in phase I/II/III clinical trials for various types of solid and haematological cancers. To date, Alisertib has been formulated and administered either as Powder in Capsule (PIT) or Enteric Coated Tablets (ECT), for oral administration to demonstrate its systemic effect. However, reported severe haematological side effects in a relatively high percentage of the patients show a clear rationale for assessing alternative routes of administration, when possible.

For cervical cancer, it is well-known that the majority of cervical cancer cases (75%) are diagnosed at an early stage, being stage I or II when the cancer is still

confined to either the cervix or the vaginal tract, owing to the increasing access to screening programs for eligible women. Therefore, intravaginal delivery systems of Alisertib offer a promising approach to treatment, as the direct contact between the cervix and the vaginal lumen makes it a feasible way to deliver treatment.

In this study we report on the design and manufacturing of mouse-sized matrix-type silicone intravaginal rings, in addition to testing and optimising their release profile *in vitro* and assessing their safety *in vivo*.

Methodology:

Pre-manufacturing viability tests were carried out on cervical cancer cell line (CaSki) to ensure the tolerability of Alisertib to both the acidity of the vaginal tract and the conditions of embedding process into silicone (elevated temperature). Following that, a High Pressure Liquid Chromatography (HPLC) method was developed to measure the release of the rings into the release medium.

Mouse-sized matrix-type silicone intravaginal rings were designed, manufactured and optimised. Release was tested *in vitro* over 21 days by incubating the rings under sink conditions in 50% isopropanol/water, and safety was evaluated *in vivo* in mice, by implanting the rings for one week and then sectioning the vaginal tract to investigate for any indications of inflammation.

Results:

Alisertib was identified as stable in all conditions related to the vaginal conditions and the manufacturing process. *In vitro*, the silicone rings maintained continuous release of Alisertib over three weeks and exhibited root time kinetics. *In vivo*, mice

vaginal tract sectioning showed no indication of inflammation (elevated neutrophils) related to the local administration of the drug over one week.

Conclusions:

The ability to deliver Alisertib to the cervix over extended period of time is an exciting opportunity to improve the therapy outcomes for cervical cancer. More precisely, these rings will aim to induce a complete regression of cervical cancer cells when treated at early stages (Stage I and II). For more advanced cases, this treatment will be applied as neoadjuvant treatment and/or as follow-up treatment after surgery and radiotherapy to prevent recurrence of the disease. Moreover, local delivery will aid in reducing the required drug dose, avoiding the first pass metabolism, and reducing the systemic side effects of the drug.

These results support taking this platform a step forward to testing in animal models like sheep, and further to clinical trials.

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Chapter 1: Introduction and Literature Review

1.1. Cervical Cancer

Cervical cancer was responsible for claiming more than 265,000 lives worldwide in 2012, and another 520,000 cases were diagnosed in the same year. In contrast with other varieties of cancer, this cancer lethality was ranked fourth of all cancer deaths among women globally (1).

Distribution of the disease varied significantly over different regions of the world. Approximately, 85% of the global burden was diagnosed in developing countries and 87% of all deaths related to cervical cancer were recorded in these regions. This is believed to be due to the screening programs and HPV vaccination that are utilised in the developed countries (1-3).

The highest risk areas included Eastern Africa and Melanesia, followed by Southern and Middle Africa, with the lowest rates in Australia/New Zealand and Western Asia (1-3). In Australia, it is estimated that 903 females will be diagnosed with cervical cancer during 2016, with 250 deaths as a result (4).

1.1.1. Human Papillomavirus (HPV) is the Causative Factor of Cervical Cancer

HPV is considered one of the major contributors to infection-related cancer worldwide, and is responsible for 5% of the entire cancer onus (5, 6). Some of the cancers that HPV infection can contribute to include head and neck cancer, penile carcinoma, anal carcinoma, vulvar carcinoma and vaginal carcinoma (6).

Cervical cancer is the most common HPV-related disease. Infection is usually acquired sexually and HPV is found in more than 99% of cervical cancer cases.

Therefore, it is now accepted that HPV is the key risk factor for and the definitive cause of cervical cancer (3, 6-10). Nevertheless, not all cases of infection with HPV develop to malignancy.

Infections with HPV are common; between 50-80% of sexually active women will become infected during their lifetime (11-13). Luckily, most of the cases resolve spontaneously or only cause minor infections that are easy to treat. Warts, anogenital condyloma, and oro-laryngeal papillomatosis are some of the diseases caused by this virus, which require simple treatment to be resolved (3). Within two years of infection, approximately 90% of all HPV infections are cleared, and only a small proportion persists, that may then develop into malignant lesions within 10-30 years (figure 1.1) (14). To be able to develop to malignancy, highly persistent viruses are required, which is mainly a function of the type of the HPV (15).

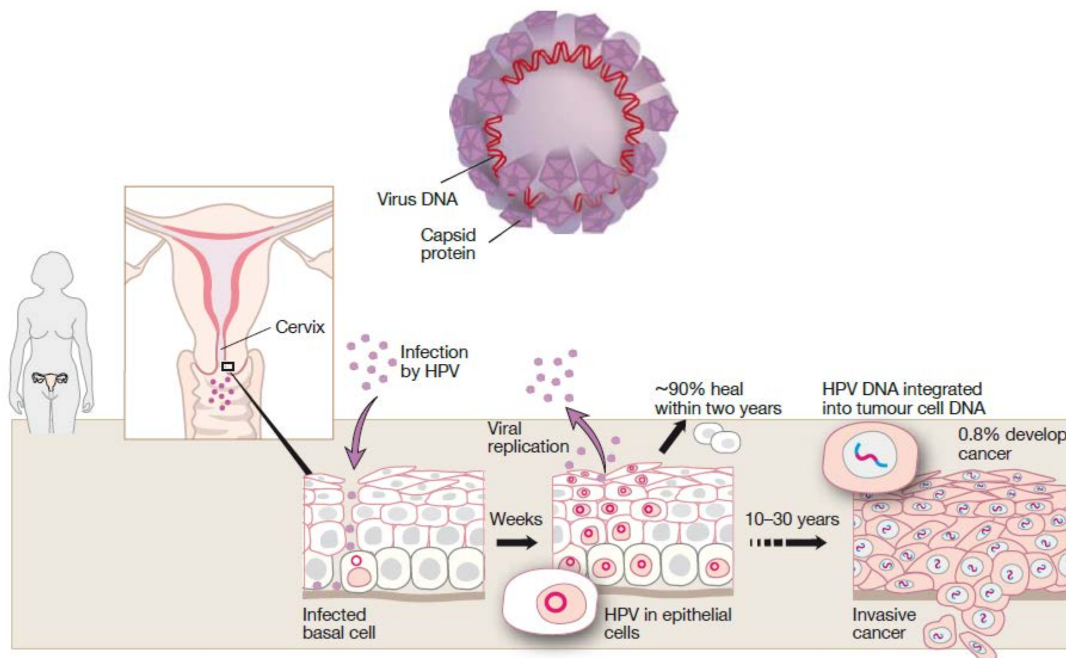


Figure 1.1. The development of cervical cancer; from the early HPV infection to invasive cancer (adopted with a permission[1] from Andersson, 2008 (16))

[1] © The Nobel Committee for Physiology or Medicine 2008, Illustration: Annika Röhl

1.1.1.1. Types of HPV and their Potential to Cause Cervical Cancer

More than 80 genetic types of this double-stranded DNA virus have been identified, which have the ability to infect various tissues in the human body. However, two genetically diverse subtypes (HPVs types 16 and 18) cause more than 84% [(15)] of invasive cervical cancer cases [(7, 15, 17-19)].

The potential of these subtypes to be oncogenic is mainly driven by the proteins expressed by HPV E6 and E7. The combination of these two gene products aids in repressing apoptosis and maintaining the S-phase state of the infected cells [(15, 20)].

1.1.1.2. The Carcinogenic Roles of HPV E6 and E7

HPV E6 protein consists of approximately 150 amino acids with two zinc-finger motifs [(21, 22)]. The main carcinogenic effect of E6 is driven by its ability to block apoptosis and prolong the lifespan of infected cells. This effect is mediated by blocking p53 from entering the nucleus and performing its role in apoptosis. The absence of p53 from the nucleus allows cells to divide continually despite the damage that has occurred in their DNA [(23, 24)]. The effects of HPV E6 are further assisted by HPV E7.

HPV E7 protein performs its roles inside the nucleus, where it interacts with many factors responsible for regulating cell growth. By binding to these factors, E7 transforms the infected cells and extends their life span [(25, 26)]. Furthermore, E7 aids in degrading the retinoblastoma protein which inhibits the retinoblastoma tumour suppressor pathways [(27, 28)]. The synergetic effects of HPV E6 and HPV E7 are believed to immortalize infected cells and support the high resistance of the virus; these interactions occur during many stages of HPV life cycle, from infection through to developing a malignancy [(29)].

1.1.1.3. HPV Life Cycle: From Early Infection to the Late Stages

HPV targets basal epithelial cells via its receptor, the alpha 6 integrin [30], where it employs the cellular replication mechanism to synthesize low levels of viral DNA. In this process, around 50-100 genomes are synthesised per cell. The generated genomes amplify to more than 1000 copies during the differentiation of the infected cells, and capsid proteins are expressed during the same stage. Infectious virions are then built and released [31].

The replication of the viral episome depends on many factors in the host cells that are not expressed beyond the S-phase. HPV overcomes this challenge by inducing S-phase in the infected cells without preventing them from differentiating [25, 31].

HPV E6 and E7 take a major place in this process, where E6 is responsible for inhibiting cell cycle arrest mediated by p53 while E7 inhibits retinoblastoma tumour suppressor pathways and supports replication of the viral DNA. The latter is achieved by inducing the keratinocytes of the host cell to re-enter S-phase [31].

While E6 and E7 play essential roles in the carcinogenicity of HPV, other factors potentiate its efficacy and increase its chances of initiating immortality in cells that eventually develop to malignancy [32-35].

1.1.2. Risk Factors Potentiate the Malignancy

Many factors potentiate the chances of acquiring the virus or strengthen its activity to develop malignant lesions. Smoking [36], long term use of oral contraceptives [37], and HIV infection [38] are well established as cofactors in the progression of cervical cancer. In addition, probable cofactors include immunosuppression, co-infection with herpes simplex virus type 2, and *Chlamydia trachomatis* infection. On the other hand,

some practices are found to provide a protective role against the malignancy; following a diet rich with fruits and vegetables being a notable example [32-35].

To sum up, the continuous and uncontrolled growth in the tissue of the cervix that result in cervical cancer is induced by persistent untreated infection with Human Papillomavirus (HPV) [1, 39, 40], and potentiated by other co-factors such as multiple sex partners, smoking and long term use of oral contraceptives [32-35]. On the other hand, some practices may prevent the progression of the disease or avoid acquiring the virus in the first place. These are screening and vaccination respectively.

1.1.3. Screening Tests Detect the Malignancy at its Earliest Stages

A considerable amount of literature has been published on the importance of regularly performing cervical cancer screening starting from the age of 21. According to recent reports, more than 50% of the cervical cancer cases could have been avoided if proper screening had been performed [41, 42].

Two types of screening tests are believed to be the most accurate and reliable. These are Papanicolaou Testing which was recommended by The American Congress of Obstetricians and Gynecologists (ACOG) for annual appointments, and Cobas HPV DNA testing which was approved by the USA Food and Drug Administration (FDA) in 2014 as a first-line screening tool for women over 25 years. The latter has the ability to show individual results for the most carcinogenic subtypes of HPV (HPV types 16 and 18) and is more reliable and easier to perform [41, 43].

The screening tests aim to make it possible to treat the disease at its earliest stages, but do not provide protection against acquiring the HPV in the first place. In order to reduce the chances of developing cervical cancer, vaccination may be feasible since the disease is primarily caused by a virus.

1.1.4. Prevention of HPV Infection is Possible

Many efforts were made to achieve reliable methods to prevent HPV infections and therefore reduce the possibility of developing cervical cancer. Research in the field of cervical cancer prophylaxis has been successful in developing many reliable vaccines for this purpose. Among FDA-approved vaccines are Gardasil, Cervarix, and Gardasil 9 which show noteworthy results in diminishing HPV infections [44, 45]. Unfortunately, these vaccines do not have the ability to treat current infections of HPV, nor do they provide a complete protection against all types of the virus [39, 40]. Thus, it is crucial to address therapies for current cases, based on the spread (stage) of the malignancy.

1.1.5. Staging of Cervical Cancer

Histologically, cervical cancer is sub-grouped into many types. One of the most common is squamous-cell carcinoma (precursor lesions classified as cervical intraepithelial neoplasia (CIN)), which accounts for approximately 85% of cervical cancer cases, followed by adenocarcinoma (precursor lesions are adenocarcinoma *in situ* (AIS)) which is responsible for another 10% of cases [46-48].

Clinically, cervical cancer falls into 4 stages (Stage I-IV) as designated by The International Federation of Gynecology and Obstetrics (FIGO) (figure 1.2). This staging system primarily depends on the degree of the malignancy spread [49].

The lesion in stage I is confined to the cervix only. This stage is further sub-staged to Stage IA and IB, based on the size of the malignancy. The cancer during Stage I can only be seen at the microscopic level. In Stage II, the cancer extends beyond the cervix into the upper vagina or para-cervical soft tissue and increases in size up to 4 cm. Further expanding of the malignant cells leads to invasion of the pelvic wall and/or the lower third of the vagina where it becomes Stage III. The lesion is classified as

stage IV when it has spread to other organs like the bladder or the rectum or even further [50, 51].

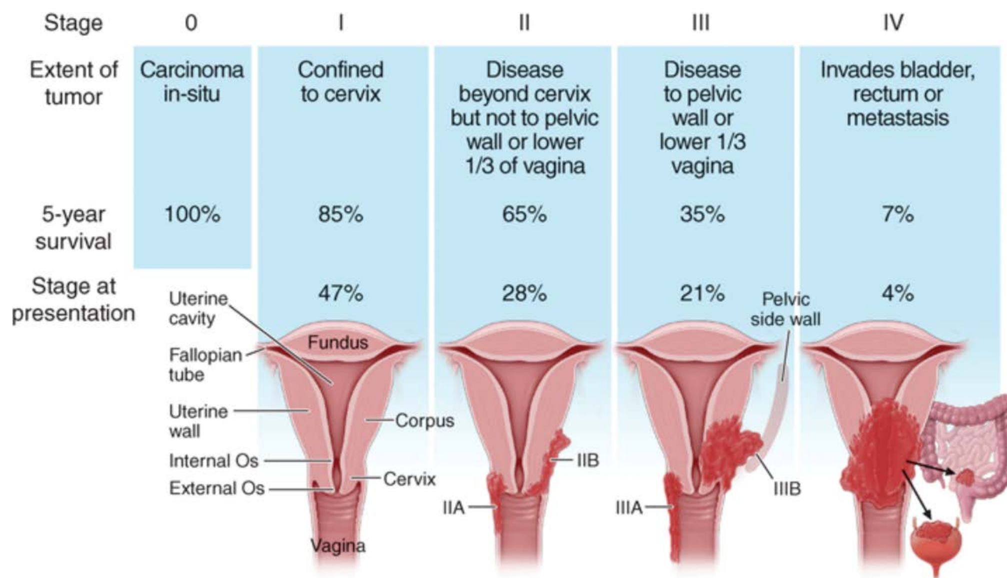


Figure 1.2. Stages of cervical cancer according to The International Federation of Gynecology and Obstetrics (FIGO) (adopted from Seiden, 2012 (51))

Staging cervical cancer at the time of primary diagnosis is a critical factor to standardize treatment options, as the choice of treatment is primarily based on the stage of the cancer [49, 52].

1.1.6. Current options for treatment

A combination of factors contributes to the choice of treatment for each individual case of cervical cancer. The stage and type of the cancer in addition to the patient's age and their preference of preserving fertility are assessed before initializing a treatment plan for each case. However, the clinical stage of the cancer is considered the benchmark for standardizing treatment [53-56].

Superficial cervical cancer in early IA stage may be treated effectively with conisation, however, radical surgery is the benchmark strategy in more advanced stages (beyond stage IA1). Such a procedure is invasive and is associated with many

adverse events [54]. These adverse events include blood loss, defects in bladder or bowel function, sexual dysfunction, and loss of fertility [54, 57]. The last one is of special concern, considering the fact that approximately 45% of women undergoing surgery in stage IB are under the age of 40 and would prefer to preserve their fertility [55, 56].

Radiotherapy is one of the first-line strategies for the treatment of cervical cancer. It is specially selected for locally advanced cases by applying External Beam Radiotherapy (EBRT) in addition to intracavitary brachytherapy [58]. Such an approach, however, is associated with serious cytotoxicity to normal tissue, which is more serious in the case of larger lesions [59].

Chemotherapeutic agents may be added to radiotherapy or used alone for cervical cancer. Available chemotherapies include cisplatin, paclitaxel, ifosfamide, epirubicin, hydroxyurea, 5-fluorouracilm and mitomycin [60, 61].

Nevertheless, the armamentarium for treatment of cervical cancer has not improved the 5-year survival rate over the last decades. During 1975-1977, the 5-year relative survival in USA was 69.1%, and this dropped by 0.3% in 35 years to reach 68.8% in 2012 [62]. In addition, the harsh and invasive side effects of these treatments significantly affect the patients' quality of life. Therefore, it can be concluded that we are in desperate need of novel strategies for the treatment of cervical cancer.

1.2. Depleting Aurora A Kinase Emerged as a Powerful Tool in Targeting E7-Expressing Cells

HPV virus is dependent on E6/E7 expression to survive. Depletion of the expression of these genes by short hairpin RNA has been shown to be sufficient to result in specific cell death of cervical cancer cell lines via apoptosis [63]. Therefore, it

was hypothesized that drugs targeting expression of E6/E7 would have high selective lethality for HPV-driven cancers such as cervical cancer [40].

1.2.1. Identifying Aurora A Kinase as a Target in Cervical Cancer

A recent functional genomics screening was performed using an siRNA library for Protein Kinases that targets 779 genes, to identify any genes whose their knockdown would result in selective lethality for an HPV-driven cervical cancer cell line (CaSki), but not for a non-HPV cell line (C33A) [40].

Depletion of Aurora A or Aurora B kinases was identified as top hits for selectively killing HPV-positive cell lines. However, the use of the Aurora B inhibitor ZM447439 has not shown *in vitro* selectivity. Therefore, the selective Aurora A inhibitor Alisertib (MLN8237) was assessed [40].

1.2.2. Sensitivity to Depleting Aurora A kinase is a Result of Addiction to E6/E7 Genes in HPV-Positive Cell Lines

Interestingly, HPV-positive cervical cancer cell lines showed high sensitivity to treatment with Alisertib [40]. In contrast, this sensitivity was not reflected in HPV-negative cell lines like C33A, which aligned with the expected outcome. Moreover, oral treatment for an animal model of cervical cancer resulted in a complete regression of the malignancy in HPV-positive cancers [40]. Surprisingly, transfecting HPV-negative cell lines with the HPV16 E7 oncogene resulted in sensitivity to Alisertib [40]. These results correlate the expression of E7 with the sensitivity to depleting Aurora A kinase which has many important roles in cell mitosis [40, 64, 65].

1.2.3. Aurora A Kinase is an Established Target in Other Types of Cancer

Aurora A Kinase is associated with many roles in cell mitosis. This includes its involvement in maturation and separation of the centrosome, cytokinesis, mitotic entry, chromosome alignment during metaphase, and bipolar-spindle assembly [(64-66)]. Interestingly, there is a growing body of literature that recognises the overexpression of Aurora A kinase in many types of cancer, which indicates its importance in the progression of these cancers [(67-69)]. For cervical cancer, depletion of Aurora A kinase or the gene encoding it (AURKA) resulted in a lethal effect on HPV-positive or HPV E7-transfected cell lines while retaining viability in normal cells and HPV-negative cell lines [(40)].

Accordingly, many AURKA inhibitors were developed. During the last 10 years, more than 13 different inhibitors of the Aurora kinases have been tested in phase I clinical trials in patients with several hematologic and solid tumours. The majority of these agents were inhibitors of all Aurora-A, -B, and –C indiscriminately. However, only few inhibitors made it through to phase II clinical trials. Several trials were suspended or not completed due to serious cytotoxic side effects observed upon administration of the clinically effective doses, while others were discontinued due to inadequate anti-tumour activity [(70-72)].

Despite the fact that there were several proposed selective inhibitors for AURKA, such as MLN 8054 [(73)], TAS-119 [(74)], MK-5108, ENMD-2076, and MLN 8237 [(75)], only MLN 8237 (also known as Alisertib) was able to progress to phase III evaluation [(76)]. Alisertib was developed as a structurally improved generation of a previously established inhibitor AURKA MLN 8054. The latter was the first AURKA inhibitor

to be evaluated in human phase I clinical trials due to its encouraging preclinical pharmaceutical characteristics such as reliable absorption and prolonged half-life. However, upon clinical administration, patients suffered from central nervous system (CNS) side effects due to its structural similarity to the benzodiazepine family. Somnolence due to GABAA α-1 benzodiazepine off-targeting was a major safety concern [(77)]. Subsequently, a change to the structure of MLN 8054 led to the development of MLN 8237 (apyrimidobenzazepines), which showed less affinity to GABAA α-1 and therefore, fewer CNS side effects [(78-84)].

1.3. Assessing the Safety of Systematic Administration of Alisertib (MLN8237): Review of the Clinical Data

Despite the reported high efficacy of Alisertib on HPV-positive cancers, *in vitro* and *in vivo*, its safety profile is still debatable. Serious side-effects related to the systematic administration of the molecule, such as neutropenia and anaemia, have been reported in Phase I/II clinical trials [(82, 85-90)]. Therefore, this section assessed the safety profile of Alisertib when administered systematically, using clinical data available as outlined in Appendix A.

1.3.1. An insight into the anti-tumour activity of Alisertib observed on various other cancers in Phase I/II clinical trials

In order to evaluate the clinical effect of Alisertib, various drug doses and formulations were tested in patients with advanced tumours. In most phase I or II trials, the clinical effect was described according to the evidence of Aurora A Kinase inhibition, achieving a clinical response, which was assessed by validated scales, time to disease progression, progression-free survival, and the duration of disease stability after the administration of Alisertib [(82, 87-89, 91)].

Although the primary end-point in the included studies was to assess the toxicity and the safety of administration of Alisertib, the reported improvement in the clinical response was promising. For example, a study by Melichar *et al* (2015), which utilized the revised version of Response Evaluation Criteria in Solid Tumors (RECIST) scale to assess the clinical response to treatment [(92)], reported a partial response rate of 18% in patients with breast cancer, and a 21% response rate in patients with small-cell lung cancer when Alisertib was administered in 21-day cycles, at a 50 mg per dose, twice daily, for 7 days followed by a 14-day washout period (the mean duration of response was 5.6 and 4.1 months, respectively) [(91)].

Similarly, Dees *et al* (2012) utilized the RECIST guidelines (the older version) [(93)], to assess the effect of this treatment on various refractory-to-standard-therapy, metastatic solid tumours, and reported a partial response rate of 23%, which was sustained for more than 3 months [(82)]. Alisertib treatment also seemed effective when administered to various haematological malignancies, with partial responses varying between 13% and 27% of the enrolled patients for at least 2 months [(87-89)].

Goldberg *et al* (2014) described the requirement of multiple treatment cycles to demonstrate the anti-tumour effect of Alisertib [(89)]. After four cycles, Alisertib treatment resulted in a median of 51 days of progression-free survival in patients diagnosed with acute myelogenous leukaemia (AML) [(87)].

In addition, the anti-tumour activity of Alisertib was also assessed according to the inhibition of Aurora A Kinase in the skin and tumour specimens. Cervantes *et al* (2012) demonstrated an Alisertib-exposure-dependent increase in the number of mitotic cells with characteristic spindle abnormalities, which supported AURKA inhibition in tumour biopsies [(90)]. Dees *et al* (2012) also collected skin biopsies pre-

treatment and 6 and 24 hours after treatment. The results showed a significant increase in the mitotic cells in the basal epithelium following treatment, reflecting a consistent AURKA inhibition by Alisertib [82].

However, data showed that the AURKA inhibition might not correlate with achieving a clinical response in some patients [87]. The latter evidence highlights the possibility of a multi-factorial effect and the involvement of multiple molecular mechanisms to achieve a clinically quantifiable effect. In other words, the inhibition of AURKA may not be enough to achieve the desired anti-tumour effect in certain types of cancers.

1.3.2. Safety Assessment and its Implications

Phase I studies [82, 87, 90] were mainly interested in determining dose-limiting toxicities (DLTs) and maximum tolerated dose (MTD) of Alisertib, and subsequently suggest a recommended phase II dose (RP2D) and treatment cycle. The three papers reported neutropenia as the highest frequent DLT, followed by other haematological side effects as leukopenia, anaemia, and thrombocytopenia. Kelly *et al* (2014) suggested that administering higher daily doses of Alisertib within a shorter period of treatment (7d) is more tolerable than longer treatment cycles and will result in fewer adverse events. Dees *et al* (2012) and Cervantes *et al* (2012) results aligned with this finding and they therefore abandoned dosing schedules of more than 7 days. It was also found that CNS side effects are related to high, once-daily doses, and dividing the dose into twice a day schedules decreased both the frequency and severity of these adverse events. Interestingly, 50 mg twice a day for 7 days of treatment followed by 14 days of recovery in 21-day cycles was suggested by the three studies as RP2D.

Kelly *et* al (2012) and Dees *et al* (2012) were also interested in comparing two oral formulations of Alisertib, namely, powder in capsule (PIC) and enteric-coated tablets (ECT). Both studies reported similar bioavailability and pharmacokinetics properties for both PIC and ECT. However, Kelly *et al* (2012) suggested ECT for future development, as it allows Alisertib to bypass the acidity of the stomach and delay dissolution to the delivery site in the small intestine.

Overall, 630 patients were assessed in the 7 studies. Only severe, life-threatening and fatal adverse events (grade ≥3 according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE)) related to the systematic administration of Alisertib were extracted individually from each study and summarized in table 1.1.

Table 1.1. Most frequently reported drug-related grade ≥3 adverse events in all papers (Papers ID as in table A.3). All adverse events and serious adverse events were graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE), The values in the table represent n(%) and is visually grouped according to the percentage of occurrence as follows: 1%-9%, 10%-19% and ≥20%.

Study ID (n) / **Side Effect**	**1 (n=72)**	**2 (n=249)**	**3 (n=58)**	**4 (n=48)**	**5 (n=57)**	**6 (n=87)**	**7 (n=59)**	**Total (n=630)**
Neutropenia	30 (42%)	107 (43%)	26 (45%)	30 (63%)	7 (12%)	26 (30%)	20 (34%)	246 (39%)
Leukopenia	16 (22%)	53 (21%)	14 (24%)	26 (54%)	2 (4%)		13 (22%)	124 (20%)
Anaemia	10 (14%)	26 (10%)	11 (19%)	17 (35%)	5 (9%)	7 (8%)	6 (10%)	82 (13%)
Thrombocytopenia	10 (14%)	18 (7%)	16 (28%)	16 (33%)	5 (9%)	5 (6%)	7 (12%)	77 (12%)
Fatigue and Asthenia	3 (4%)	15 (6%)	2 (3%)	4 (8%)	7 (12%)	3 (3%)		34 (5%)
Stomatitis		19 (8%)	1 (2%)	7 (15%)	2 (4%)	1 (1%)	4 (7%)	34 (5%)
Febrile neutropenia	7 (10%)		5 (9%)	6 (13%)	6 (11%)	7 (8%)		31 (5%)
Lymphopenia	7 (10%)		4 (7%)	2 (4%)			4 (7%)	17 (3%)
Diarrhoea	1 (1%)	3 (1%)	1 (2%)	1 (2%)	1 (2%)	6 (7%)		13 (2%)
Mucositis oral	9 (12%)							9 (1%)

Somnolence		3 (1%)		1 (2%)	2 (4%)	2 (2%)		8 (1%)
Nausea	2 (3%)	2 (1%)		1 (2%)		1 (1%)		6 (1%)
Vomiting	1 (1%)	3 (1%)				1 (1%)		5 (1%)
Palmar-Plantar Erythrodysesthesia Syndrome	3 (4%)							3 (<1%)
Pneumonia				3 (6%)				3 (<1%)
Pancytopenia				3 (6%)				3 (<1%)
Sepsis	1 (1%)				1 (2%)			2 (<1%)
Decreased Appetite		1 (1%)		1 (2%)				2 (<1%)
Dehydration				2 (4%)				2 (<1%)
Leukocytosis	1 (1%)							1 (<1%)
Anal mucositis	1 (1%)							1 (<1%)
Typhlitis	1 (1%)							1 (<1%)
Lung infection	1 (1%)							1 (<1%)
Alanine aminotransferase increased	1 (1%)							1 (<1%)
Aspartate aminotransferase increased	1 (1%)							1 (<1%)
Skin and subcutaneous tissue disorder Other	1 (1%)							1 (<1%)
Hypertension	1 (1%)							1 (<1%)
Confusion				1 (2%)				1 (<1%)
Dyspnoea				1 (2%)				1 (<1%)

The single most striking observation from the safety data was the haematological disturbance in a relatively high percentage of the patients, which aligns with the characteristics of cytotoxic agents [(94)]. On top of the list, severe neutropenia occurred in 246 (39%) patients, followed by Leukopenia, which was reported in 124 (20%) patients. Severe anaemia and thrombocytopenia were reported in 13% and 12% of the

patients, respectively. Less frequent severe side effects included fatigue, stomatitis and febrile neutropenia and were reported in 5% of the total number of patients.

The safety profile of the systemic administration of Alisertib is worrisome. Given its cytotoxic nature, one would expect that Alisertib would cause these serious side effects. In order to manage these effects, long recovery periods were required (7 days of treatment followed by 14 days for recovery), which may limit the drug's efficacy. Therefore, exploring alternative routes of administration when possible could be the answer. Cervical cancer is a great example, knowing that inhibiting Aurora A kinase was established as a strong potential target in this cancer [(40)]. Moreover, it is well-known that the majority of cervical cancer cases are diagnosed at an early stage, being stage I or IIa when the cancer is still confined to the cervix, owing to the increasing access to screening programs for eligible women [(51)]. Intravaginal delivery systems are therefore a strongly recommended alternative.

1.3.3. Localizing the Treatment of Alisertib for Cervical Cancer: What to Expect?

This topic can best be treated under three headings: safety, efficacy, and technical challenges. Firstly, questions have been raised about the safety of the systemic administration of Alisertib. Clinical trials undertaken on this molecule have shown serious haematological adverse events in a relatively high percentage of the patients [(section 1.3.2)]. Localizing the administration of Alisertib in cervical cancer is therefore suggested to reduce/avoid any serious systemic toxicity. On the other hand, possible safety issues related to the local administration in the vaginal tract should be investigated. This includes any negative alteration of the vaginal environment or harsh effects on the epithelium.

Secondly, the direct delivery of Alisertib to its site of action in cervical cancer is expected to be significantly more effective at relatively lower doses than systemic delivery. However, this approach targets local stages of the diseases, and systemic delivery should be considered in the advanced stages of the malignancy.

Thirdly, the characteristics of the drug itself, in addition to the length of treatment, should be considered when developing a topical carrier in the vaginal tract. In other words, the physiochemical characteristics of Alisertib (including the high hydrophobicity (logP=5.74, as predicted using MarvinSketch Software, ChemAxon)), and the expected long duration required for treatment, both limit the flexibility of developing an adequate topical drug delivery platform.

1.3.4. Choosing a Platform for Delivery

To date, a large variety of topical delivery systems for vaginal applications have been developed. Such delivery systems include liquids (solutions and suspensions), semi-solids (creams, ointments and hydrogels) and solids like tablets, capsules and suppositories [(95, 96)].

All forms mentioned so far, however, are not able to maintain a drug release for more than 1-2 days. Furthermore, many of the previously mentioned systems are messy and not convenient for patients' use [(97, 98)].

In contrast, intra-vaginal rings are convenient for self-administration over an extended period of time, which results in increased adherence. In addition, the various materials used in their manufacture provide a degree of flexibility to carry the drug of interest. Moreover, high reliability and a good safety profile were achieved by those currently commercially available [(99-104)].

1.4. Intra-Vaginal Rings for Delivering Alisertib

Intra-Vaginal Rings (IVRs) represent a topical delivery system for female genital disorders that sit in the cervical area when administered. IVRs diameter is usually between 50 and 75 mm, and their cross-sectional diameter is 4-9.5 mm. They are flexible, torus in shape, elastomeric drug carriers that maintain long term delivery (figure 1.3). Their ease of self-application and long term delivery avoid the need for a healthcare professional and were reported to increase patients' compliance, resulting in a significant improvement in adherence [99, 101, 105-109].

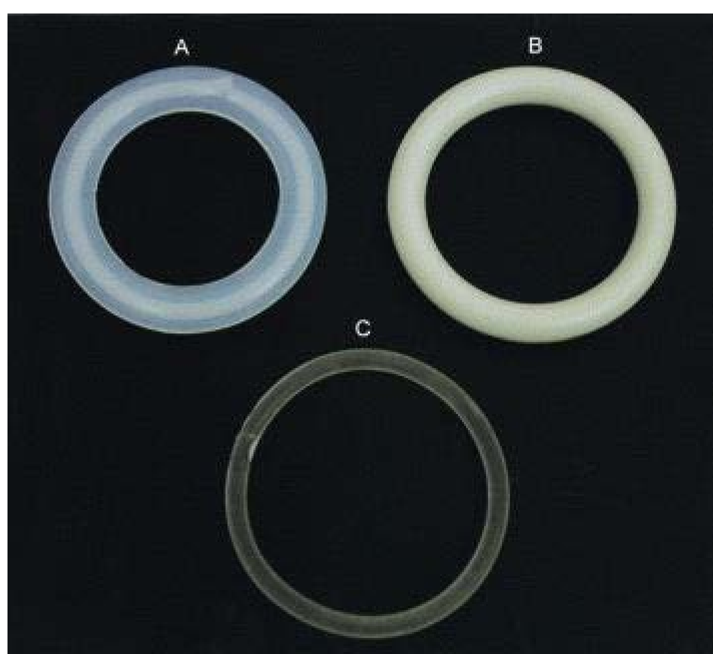


Figure 1.3. Commercial FDA approved intra-vaginal rings (A) Estring® (estradiol intra-vaginal ring, Pfizer) fabricated with silicone (B) Femring® (estradiol acetate intra-vaginal ring, Warner Chilcott) Fabricated with silicone (C) NuvaRing® (etonogestrel/ethinyl estradiol intra-vaginal ring, Organon) fabricated with EVA (Reprinted with permission from Alexander, Baker, 2004 (110))

1.4.1 The Potential of IVRs in Delivering Substances to the Cervix: A Glance at the Anatomical Structure

Anatomically, the cervix is the lowest third of the uterus. In the human, the cervix is conical in shape, and its dimensions are 3-4 cm long and 2.5 cm wide. It is the connecting organ between the upper parts of the female reproductive system opening

in the uterus via the internal orifice and the lower parts of its opening via the external orifice into the vaginal tract. Therefore, the cervix may be divided in terms of anatomy into the super-vaginal section and the vaginal section [111-114]. This direct contact between the cervix and the vaginal tract makes it a feasible target for topical drug delivery, especially for IVRs that sit around the cervix [104-106].

The direct contact of the cervix with the vaginal lumen makes it a feasible target of topical delivery through the vagina. Interestingly, vaginal rings normally sit around the cervix and release the active substances in that area [105], which supports their potential to deliver anti-cancer substances for the treatment of cervical cancer. However, many conditions should be taken into consideration prior to the development of this platform to minimize ring-related adverse events. This includes their sizes, physical properties and their materials' biocompatibility.

1.4.2. Polymers used for manufacturing IVRs

Polymer choice is crucial in designing IVRs. Safety and mechanical properties, in addition to polymer-drug-compatibility, must be taken into consideration. The polymer to be used has to show biocompatibility and avoid toxic or allergic reactions to human tissues. In addition, flexibility for ease of insertion yet sufficient rigidity to persist in the vaginal cavity are also to be considered. IVR's polymers are usually hydrophobic in nature. Thus, the criteria of polymer choice also includes the degree of drug solubility through the polymer [98].

- **Thermoplastic and thermosetting polymers**

Polymeric materials already in use are classified into thermoplastic polymers and thermosetting polymers.

Polyethylene vinyl acetate (pEVA) and polyurethanes (figure 1.4) are examples of thermoplastic elastomers. Both polymers are processed by hot melt extrusion which is convenient for thermally-stable drug compounds. The pEVA was used to design the commercially available Nuvaring IVR. Several previous studies have demonstrated convenient release and efficacy for IVRs fabricated from polyurethanes [(115-119)].

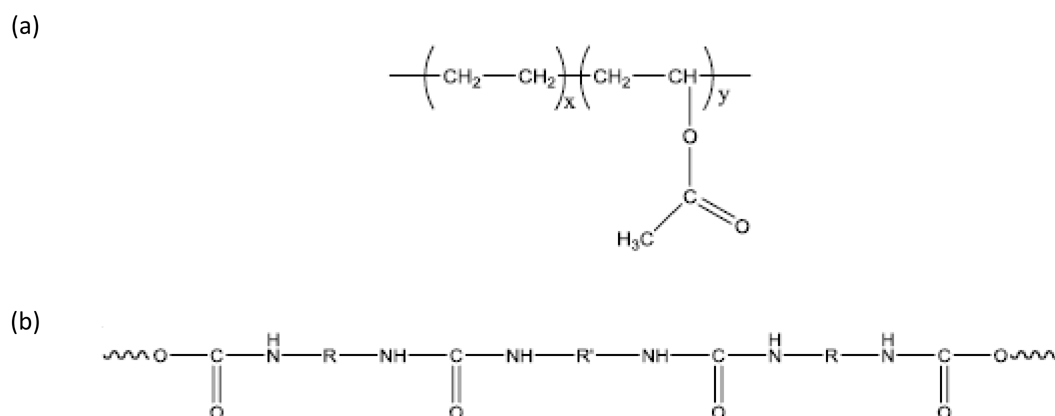


Figure 1.4. Thermoplastic Elastomers for manufacturing intra-vaginal rings (a) Polyethylene vinyl acetate (pEVA) structure. (b) polyurethane structure

On the other hand, silicon elastomers are an example of thermosetting elastomers. They are prepared by cross-linking functional linear silicone (figure 1.5) using injection moulding techniques [(120)].

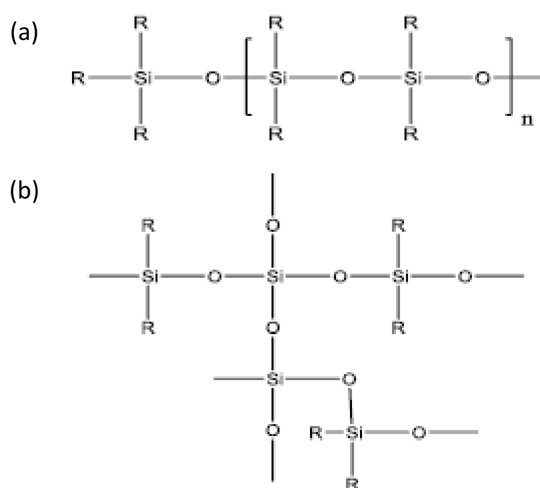


Figure 1.5. Linear and Cross-linked silicone (a) Linear silicone. (b) Cross-linked silicone.

Silicone elastomers are the most common carriers used in IVRs. Femring, Estring, Progering, and Fertiring are examples of commercially available silicone IVRs [98, 121, 122].

In addition, a large and growing body of literature has investigated microbicides, contraceptives and anti-HIV agents when applied via silicon-based IVRs, and these have demonstrated superior efficacy and convenient release profiles [123-130]. The characteristics of IVRs are also based on the manufacturing technique, where many types of IVRs are identified, including matrix, reservoir and shell-types.

1.4.3. Types of IVRs

Many designs for IVRs were developed to meet various intended release profiles and to comply with the physical and chemical properties of the drug-carrier complex. Figure (1.6) illustrates the most common types used of IVRs.

Matrix-type IVRs (figure 1.6.a) represent the simplest and most common technique. They may contain one or more drugs dissolved homogenously throughout the carrier. Drug release from matrix-type IVRs follows the Higuchi model where the release amount is inversely proportional to the square root of time [100, 104, 123, 131].

Reservoir-type and shell-type IVRs provide constant drug release rates following zero-order kinetics over long durations. In the reservoir-type (figure 1.6.b) the drug-loaded core is encapsulated in a drug-free membrane, which is responsible for the control of the release rate. On the other hand, in shell-type IVRs (figure 1.6.c), the drug-loaded layer stands between an outer drug-free membrane and an inner drug-free core. However, both of the two techniques provide lower rates of drug release than the matrix-type and are limited by their manufacturing complexities [100, 105, 127, 132].

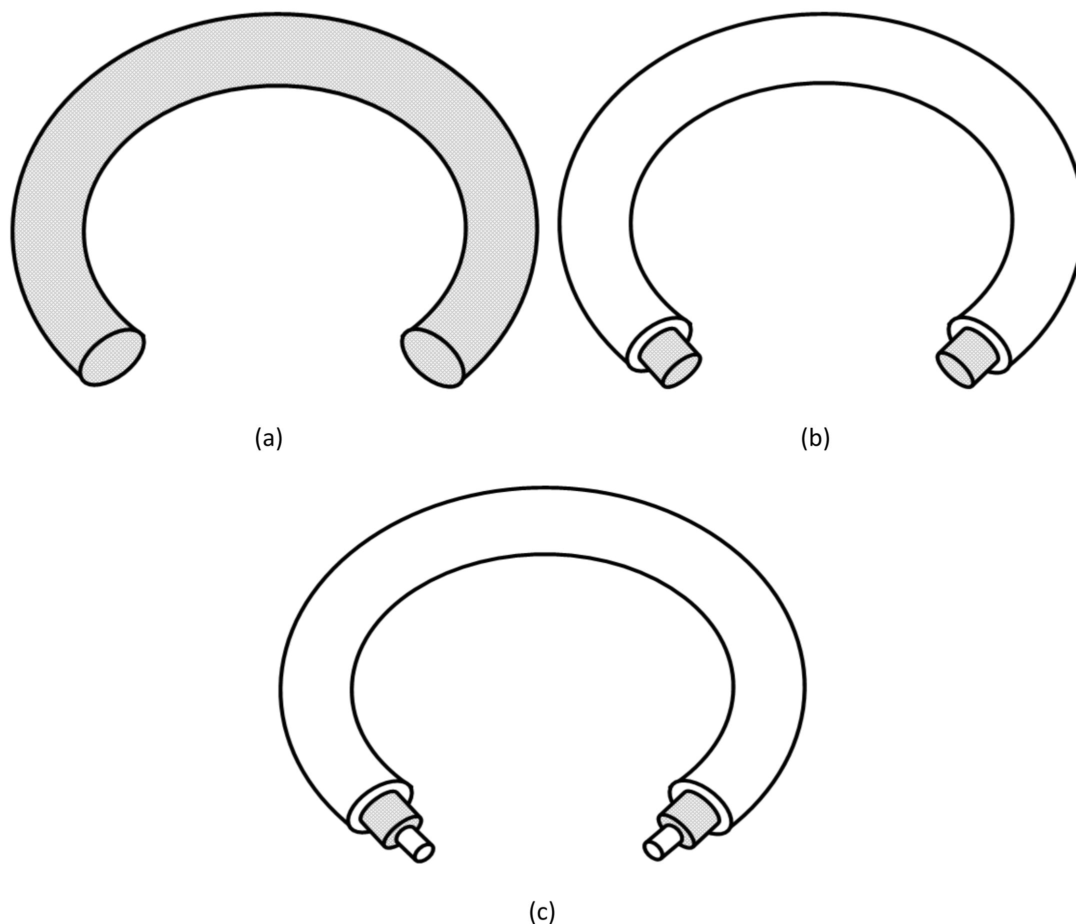


Figure 1.6. Frequent IVRs Types (a) Matrix-type (b) Reservoir-type (c) Shell-type

The present research explores, for the first time, the potential of delivering Alisertib topically through the vaginal tract to treat cervical cancer. Matrix type silicone-based Intra-vaginal rings were chosen as a starting point for their suitability with Alisertib, their safety and their expected favourable release profile. To our best knowledge, this is the first research to explore the localizing the treatment of cervical cancer using the novel Aurora A kinase inhibitor Alisertib (MLN8237), which provides an exciting opportunity to advance our knowledge in topical anti-cancer therapy.

Aims & Objectives

Hypothesis: Extended topical delivery of Alisertib (MLN8237) as a novel approach in the treatment of cervical cancer by Aurora A kinase inhibition is feasible and safe to be administered.

The overall aim of this research project is to develop, for the first time, a topical delivery system (Intra-vaginal ring) containing Alisertib, and to assess the safety of its application in mice.

The specific experimental aims and objectives are:

1. To perform pre-manufacturing tests on Alisertib, in order to ensure its efficacy and stability in the manufacturing and the application process.
   - Firstly, the IC_{50} of Alisertib on cervical cancer cell line (CaSki) will be determined.
   - Secondly, exposure to artificial vaginal fluids will be performed to assess its suitability to be applied intra-vaginally.
   - The final pre-manufacturing test will be to assess the thermostability of Alisertib at 80°C to determine the proper manufacturing process to be applied.
2. To develop a method of quantification for Alisertib using High Pressure Liquid Chromatography (HPLC) that allows selective measurement of Alisertib in various solutions.

- A method of quantification will be developed first in an organic solvent (Acetonitrile), which will allow the generation of a standard curve for quantification in other solvents.
- The method will be validated on 50% isopropanol/water in addition to simulated vaginal fluid, as these solvents will be used as the release medium for the developed silicone rings.

3. To design, manufacture, and optimise mouse-sized silicon matrix-type intravaginal rings (IVRs) that release Alisertib over a period of three weeks.
4. To characterise the *in vitro* release profile of the drug-loaded IVRs.
    - Firstly, the rings will be incubated in three ml 50% isopropanol/water, at 37°C in an orbital shaker, shaking at 60 Round per Minute (RPM).
    - Secondly, the release medium will be replaced with warmed fresh medium daily for the first five days, and then every two days until day 21.
    - The released amount of Alisertib will be measured using HPLC after each replacement of the medium.
    - Finally, the cumulative released amount of Alisertib will be plotted against time (or square root time) to identify the release pattern.
5. To assess the safety of the drug-loaded IVRs *in vivo.*
    - *In vivo* safety tests will be carried out on FVB mice by examination of immune infiltration using histological analysis (H&E staining) at the endpoint.

Chapter 2: Materials and Methods

2.1. Cell Lines

CaSki cell line (cervical cancer metastatic cells taken from the small intestine, expressing HPV-16 genome) was obtained from ATCC and used in this study. They were confirmed as authentic by STR sequence analysis in August 2015 by the AGRF. PCR was regularly performed to confirm the expression of HPV-16 gene.

2.1.1. Cell subculturing

CaSki cells were kept at 37°C, 5% carbon dioxide and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Invitrogen, Mount Waverly, Australia) supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS) (Lonza Biowhittaker, USA) and 1% PSG (100 units/ml Penicillin, 10 µg/ml Streptomycin, 29.2 g/ml L-Glutamine in a 10mM citrate buffer) (Gibco, Life technologies, USA). Infection with mycoplasma was routinely monitored by PCR (Promega).

The cells were cultured in T-25 tissue culture flasks. Subculturing was done by rinsing the flasks twice with 10 mL phosphate buffer saline (1xPBS) (8 g/L NaCL, 0.2 g/L KCl, 1.42 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4), followed by incubating the flask with 0.5 mL 0.5% trypsin-EDTA (400 mg/L KCL, 60 mg/L KH_2PO_4, 350 mg/L $NaHCO_3$, 8000 mg/L NaCl, 90 mg/L Na_2HPO_4-$7H_2O$, 1000 mg/L D-Glucose, 200 mg/L EDTA 4Na $2H_2O$, 10 mg/L Phenol Red and 500 mg/L Trypsin) (Gibco, Life technologies, Canada) for 4 minutes to allow cells to detach. Trypsin was then deactivated by adding 2.5 mL of the complete media.

The cell suspension was then centrifuged for at 369 *x*-g for 5 minutes at 21°C, and supernatant discarded to remove any traces left of active trypsin. The pellet was

resuspended in 2.5 mL media. Finally, 0.25 mL of the suspension was added to 5 mL fresh supplemented media.

2.1.2. PCR-based detection of Mycoplasma in cell culture

Detecting Mycoplasma was performed on 70-90% confluent cells. Briefly, 100µl of media was drawn and boiled for 5 minutes to release DNA. The reaction was performed using the premixed ready-to-use solution G2 GoTaq Green Mastermix (Promega) as per manufacturer's protocol.

A reaction volume of 25µl was prepared on ice as in table 2.1:

Table 2.1. Preparation of 25µl reaction volume for Mycoplasma detection

Component	Volume
2x GoTaq G2 Hot Start Green Master Mix	12.5µl
Universal Forward primer (10µM) (Myco_Universal_F)[2]	0.5µl
Universal Reverse primer (10µM) (Myco_Universal_R)[3]	0.5µl
Boiled media sample (DNA template)	2µl
Nuclease-free dH_2O	9.5µl

Following brief mixing, the tubes were placed into a thermal cycler (Kyratec SuperCycler), and subjected to temperature cycling as per the conditions in table 2.2:

Table 2.2. PCR cycling conditions for Mycoplasma detection

Cycle Steps	Cycles	Temperature	Time
Initial Denaturation	1	95° C	2 min
Denaturation Annealing Extension	30	95° C 60°C 72°C	60 sec 30 sec 30 sec
Final Extension	1	72°C	5 min
Hold	1	8°C	∞

[2] Oligo sequence: GTGGGGAGCAAAYAGGATTAGA
[3] Oligo sequence: GGCATGATGATTTGACGTCRT

Negative control (no template) and positive control (known Mycoplasma positive control) were included for comparison. Finally, all samples were run on agarose gel as outlined in section 2.1.2.1.

2.1.2.1. Agarose Gel Preparation

All PCR samples were run on 1% agarose gel prepared by dissolving 1% agarose in Tris base/acetic acid EDTA buffer (Biorad, TAE 1x). After adding agarose to the buffer, the suspension was heated in a microwave on high for 1 minute, stirred, and then heated again for 30 seconds. Brief cooling on water was performed before pouring into gel holding racks. Ethidium bromide (sigma, 10mg/ml) was mixed into the gel (0.00625%). Finally, samples were run at 80V for 80 minutes.

2.1.3. Cryopreservation

For long term storage of cells, trypsin was used to detach cells from the flask as per the previous method before transferring the cell suspension to a 15mL Falcon tube and centrifuging at 400 *x*-g for 5 minutes at 21°C. Pellets were then resuspended in cell freezing solution (93% FBS and 7% (v/v) Dimethyl sulfoxide (DMSO)) and then transferred into cryogenic tubes (Thermo Scientific-Nalgene, New York), and stored at -80°C overnight before being transferred into a liquid nitrogen tank.

2.1.4. MTT cell- viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for cells seeded in a 96-well plate to measure viability. Briefly, media was replaced with 100 µl of fresh DMEM and 10 µL of 12mM MTT prepared in PBS (1.09 mM final concentration) was added to each well. That was followed by 4 hours of incubation in 37°C before the media was replaced with 100 µL of DMSO.

DMSO was mixed thoroughly in the wells and then incubated at 37°C for another 10 minutes. Finally, absorbance was read in triplicates at 544 nM in a microplate reader (FLUOstar OPTIMA).

2.2. Simulated Vaginal Fluid

Simulated Vaginal Fluid (SVF) was prepared following the method of Owen and Katz [133]. Firstly, 3.51g of NaCl, 1.40 g of KOH, 0.222 g of $Ca(OH)_2$, 0.018 g of Bovine Serum Albumin, 2 g of Lactic Acid, 1 g of Acetic Acid (glacial), 0.16 g of Glycerol, 0.4 g of Urea and 5 g of Glucose were mixed, then dissolved in 1 L of distilled water. Finally, the pH was adjusted to 4.2 using 0.1N HCL.

2.3. High Performance Liquid Chromatography (HPLC)

2.3.1. Absorbance Spectra of Alisertib

A stock solution of Alisertib (1 mg/mL) was prepared in 1% formic acid/acetonitrile. Lambda max was identified by scanning 200 µL of the stock solution in a 96 well-plate over wave length of 200-650 nM on Multiskan™ GO microplate spectrophotometer (ThermoFisher Scientific). As a control, 1% Formic Acid in acetonitrile was used.

2.3.2. HPLC-Analytical

Shimadzu Prominence LC-20AT coupled to a SPD-M20A Diode Array Detector was used to perform the HPLC analysis with a GraceSmart Reverse Phase C18 Analytical HPLC Column, 150mm x 4.6mm, 3µ particle size (Fisher Scientific, USA).

An injection volume of 40µl was injected through the HPLC system. A mixture of solvent A (0.1% Formic Acid in ultra-pure water (milli q)) and solvent B (0.1% Formic Acid in HPLC grade acetonitrile (RCI Labscan, Thailand)) was used to perform the

analysis. Both solvents were mixed to form varying gradient elutions, the most successful being from 50% solvent B over 0.25 minutes, gradual increase to 95% solvent B over 4.75 minutes, holding 95% solvent B for 2 minutes and returning to 50% solvent B over 0.5 minutes which was maintained for 7.5 minutes, the flow rate being a constant 1ml/min for the whole run at 30°C temperature.

A linear forced through zero calibration curve was generated using 6 standard concentrations of Alisertib (3854.2 nM, 963.54 nM, 385.43 nM, 192.71 nM, 96.35 nM and 38.54 nM).

2.3.3. LCMS - Analytical

Liquid chromatography/mass spectrometry analysis was conducted using Shimadzu UFLC/MS (Prominence UFLC high pressure gradient system/LCMS-2020 single quadrupole mass spectrometer; LCMS) to measure parent ion mass and obtain fragmentation spectrum of parent ions. Shim-pack GISS C18 (150 mm x 2.1 mm, 3 μm) column was used. The mobile phase gradient used a mixture of solvent A (0.1% Formic Acid in ultra-pure water) and solvent B (0.1% formic acid in HPLC grade acetonitrile (RCI Labscan, Thailand)). Both solvents were mixed to form varying gradient elutions, the most successful being from 50-95% solvent B over 2.5 minutes, holding 95% solvent B for 1 minute and returning to 50% solvent B over 0.01 minutes which was maintained for 2.99 minutes, the flow rate being a constant 0.375ml/min for the whole run. Injections of 10μL were made and data were acquired in both positive and negative ion modes initially.

For analysis of the SVF and blank samples, the mass spectrometer was operated firstly in “MS” mode, where samples were sprayed and ionised from the nanospray needle, then passed through the first analyser (quadrupole) which is used only as a lens

to focus the beam of ions into the time-of-flight analyser (TOF). The TOF analyser then separates the ions based on the mass-to-charge ratio (m/z), identifying the compound's exact mass with a 2ppm mass accuracy.

A blank SVF sample along with an Alisertib treated sample were analysed in order to observe new molecules formed as a result of the Alisertib treatment. Once the masses' newly identified ions in question were determined, the runs were repeated in "MS/MS" mode, or Tandem Mass Spectrometry (TMS).

In TMS analysis, ions were selected by the quadrupole analyser based on their mass and retention time, and transmitted through a collision cell which contained ultra-high purity nitrogen. Bombardment of the sample with ultra-high purity nitrogen causes the ions to fragment, and these fragments aree then detected by the TOF analyser.

2.3.4. Preparing samples containing proteins for HPLC

Proteins were precipitated in all SVF samples before they were injected through the HPLC system. Acetonitrile was used following a validated method in plasma [(134)]. Briefly, 1 part of SVF was mixed with 2 parts of acetonitrile and then vortexed immediately for 20 seconds. Samples were then settled for 20 minutes before centrifuging at 21500 *x*-g for 10 minutes at ambient temperature. The supernatant was used for HPLC analysis.

2.4. Gene expression studies

2.4.1. DNA extraction using TRIzol® Reagent

DNA extraction was performed on confluent cells incubated in T-25 flasks. Media was discarded first before cells were rinsed once with 1XPBS. One ml of Trizol reagent (Invitrogen) was added to the flask and it was rocked for 5 minutes. Next, a

cell scraper was used to scrape cells before they were transferred to a 1.7ml microcentrifuge tube, where 200µl 100% chloroform was added and the tube mixed vigorously. That was followed by 15 minutes incubation at room temperature and then centrifugation at 134 *x*-g for 15 minutes at 4°C. The top layer was discarded, and 300µl 100% ethanol was added to the remaining 2 layers (DNA interphase and pink trizol/phenol bottom layer) and mixed by inversion. The mix was incubated for 3 minutes before being centrifuged at 371 *x*-g for 5 minutes at 4°C to pellet the DNA. Phenol/ethanol supernatant was discarded and the pellet was then washed 3 times by adding 0.1M sodium citrate in 10% ethanol, incubating for 30 minutes with occasionally mixing, centrifuging at 371 *x*-g for 5 minutes at 4°C, and then removing the supernatant. The DNA pellet was then suspended in 1.5ml 75% ethanol and incubated at room temperature for 20 minutes before centrifuging at 371 *x*-g for 5 minutes at 4°C. Finally, supernatant was removed and the pellet was air-dried for 15 minutes before adding TE buffer (10 mM Tris and 1 mM EDTA, pH adjusted to 8.0 using 0.1 M HCl). The mix was stored at -20°C for longer periods.

2.4.2. Agarose Gel Preparation

All PCR samples were run on 1% agarose gel prepared by dissolving 1% agarose in Tris base/acetic acid EDTA buffer (Biorad, TAE 1x). After adding agarose to the buffer, the suspension was heated in a microwave on high for 1 minute, stirred and then heated again for 30 seconds. Brief cooling on water was performed before pouring into gel holding racks. Ethidium bromide (sigma, 10mg/ml) was added and mixed into the gel (0.00625%). Finally, samples were run at 80V for 80 minutes.

2.4.3. PCR

Conventional PCR reactions were performed using the premixed ready-to-use solution GoTaq Green Master Mix (Promega) as per manufacturer's protocol, with HPV16 (forward and reverse) primers (Sigma-Aldrich) for CaSki cells. Briefly, the GoTaqGreen Master Mix was thawed at room temperature, vortexed briefly and pulse-spun in a microcentrifuge to collect the material at the bottom of the tube. A reaction volume of 25µl was prepared on ice as in table 2.3.

Table 2.3. Preparation of 25µl reaction volume for HPV16 gene expression detection

Component	Volume	Final concentration
GoTaqGreen Master Mix 2X	12.5µl	1X
10µM forward primer[4]	0.25–2.5µl	0.1–1.0µM
10µM reverse primer[5]	0.25–2.5µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water up to	25µl	N.A.

The reactions were then placed in a thermal cycler (Kyratec SuperCycler) which was preheated to 95°C. Cycling conditions for the PCR assay were as in table 2.4. Finally, all samples were run on an agarose gel as outlined in section 2.1.2.1.

Table 2.4. PCR cycling conditions for HPV16 gene expression detection

Cycle Steps	Cycles	Temperature	Time
Initial Denaturation	1	95° C	2 min
Denaturation Annealing Extension	40	95° C 52°C 72°C	30 sec 30 sec 30 sec
Final Extension	1	72°C	5 min
Hold	1	4°C	∞

[4] Oligo sequence: GAACCGGACAGAGCCCATTA
[5] Oligo sequence: CGAATGTCTACGTGTGTGCTTTG

2.5. Pre-manufacturing Testing

2.5.1. Determining the IC_{50} (half maximal inhibitory concentration) of Alisertib

CaSki cells were seeded in a 96-well plate (10,000 cells/well) and incubated at 37°C for 4 hours to allow cells to attach before media was replaced with 100 µl of various concentrations of Alisertib-treated media. Concentrations ranged from 40mM to 40nM (40mM, 20mM, 10mM, 5mM, 2.5mM, 1.25mM, 625nM, 312.5nM, 156.25nM, 78.13nM and 39.06nM). Viability was determined for each concentration using an MTT assay after 72 Hrs of incubation in a 37°C incubator. A dose-cell viability curve was then drawn to determine the IC_{50} of Alisertib on the CaSki cell line.

2.5.2. Testing Alisertib activity following exposure to simulated vaginal fluids

One part of 50 mM drug/DMSO with was incubated with 1 part SVF for various times (1 Hr to 3 Hrs). Following this, the drug was diluted in media to obtain a final concentration of 200 mM of Alisertib and applied to CaSki cells seeded in 96-well plates at 10,000 per well for 72 hrs before MTT assays were performed as per the previous method. The same concentration of Alisertib not subjected to SVF was used as a control.

2.5.3. Testing Alisertib thermostability

Alisertib thermostability was assessed by exposing 25 mM Alisertib (dissolved in 100 % DMSO) to 80°C for 30 minutes. Following this, the heated solution was diluted into media to obtain a final concentration of 200 mM Alisertib and applied to CaSki cells seeded in 96-well plates at 10,000 per well for 72 hrs before MTT assays were

performed as per the previous method. The same concentration of Alisertib not subjected to heat was used as control.

2.5.4. Alisertib solubility in 50% Isopropanol

A suspension of 1 mg/ml (1% w/v) of Alisertib was prepared in 50% isopropanol. The suspension was vortexed for 1 minute before being sonicated in a water bath (Thermoline Scientific Ultrasonic Cleaner Set WUC-A02H) at 37°C for 10 minutes.

Sonication was followed by another minute of vortexing before being centrifugation at 21500 *x*-g for 10 minutes at ambient temperature to pellet the undissolved drug. The supernatant was transferred to a fresh tube, diluted (1 in 100) using 50% isopropanol, and then injected through the HPLC system to determine the amount of the drug dissolved.

2.5.5. Alisertib stability in the release media (SVF and 50% Isopropanol)

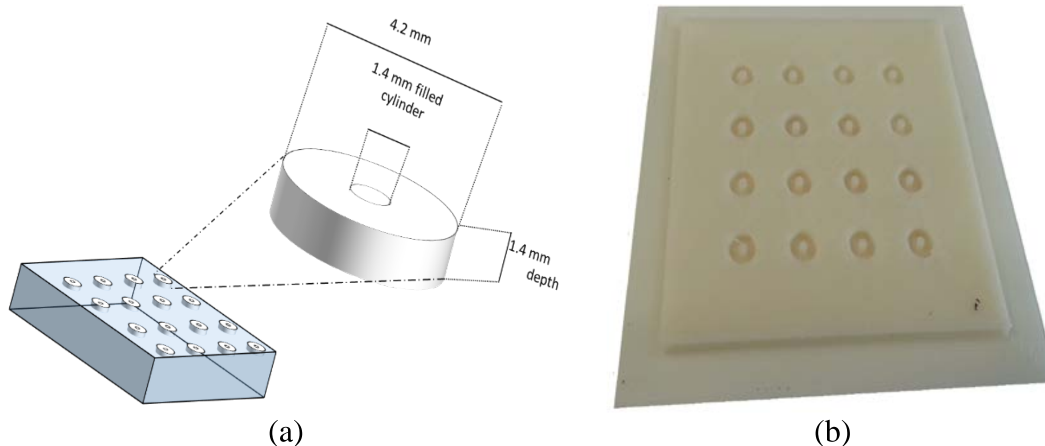
Rings were incubated in the release media for 24-48 hours before media was replaced and release was measured by HPLC.

Different concentrations of Alisertib were prepared both in SVF and 50% isopropanol and these solutions were incubated at 37°C for 48 hours. The remaining mass of Alisertib was measured at 0, 1, 2, 4, 6, 12, 24 and 48 hours using HPLC.

2.6. Ring Fabrication

2.6.1. Preparation

A biomedical grade SILASTIC MDX4-4210 silicone elastomer base (Dow Corning, Thailand) was used as a base material to manufacture the rings. Mouse-sized moulds (1.4x1.4x1.4 cm) were 3D printed as shown in figure 2.1.



(a) (b)

Figure 2.1. Mould for mouse-sized rings manufacturing (a) the initial design (b) the final mould

2.6.2. Ring Manufacture

Latex-free gloves, glass, stainless steel and plastic medical instruments were used to handle the silicone. Both the curing agent and the elastomer base were heated to 80°C to facilitate the curing process and decrease the silicone viscosity. Following that, 1 part of the curing agent was added to 10 parts by weight of silicone elastomer base and this was mixed on a glass board for 2 minutes at room temperature. Alisertib powder was ground in a ceramic mortar and pestle for 10 minutes before the required amount of Alisertib was then added and mixed thoroughly for another 10 minutes at room temperature using a stainless steel spatula.

After the mixing, the resulting blend was moved to a 1 ml syringe and injected through a 19 gauge medical needle with a removed bevel into the mould (This reduced the amount of bubbles in the blend).

2.7. Release Profile

Mouse-sized rings loaded with various concentrations of Alisertib (0.5%, 1%, 1.5% and 2%) w/w were formed and the mass of each ring was recorded.

The rings were hung in scintillation vials containing 3 ml of the release medium to maintain sink conditions (the concentration of the drug should not exceed 10% of saturation) using thin glass tubes (figure 2.2) to avoid floating.

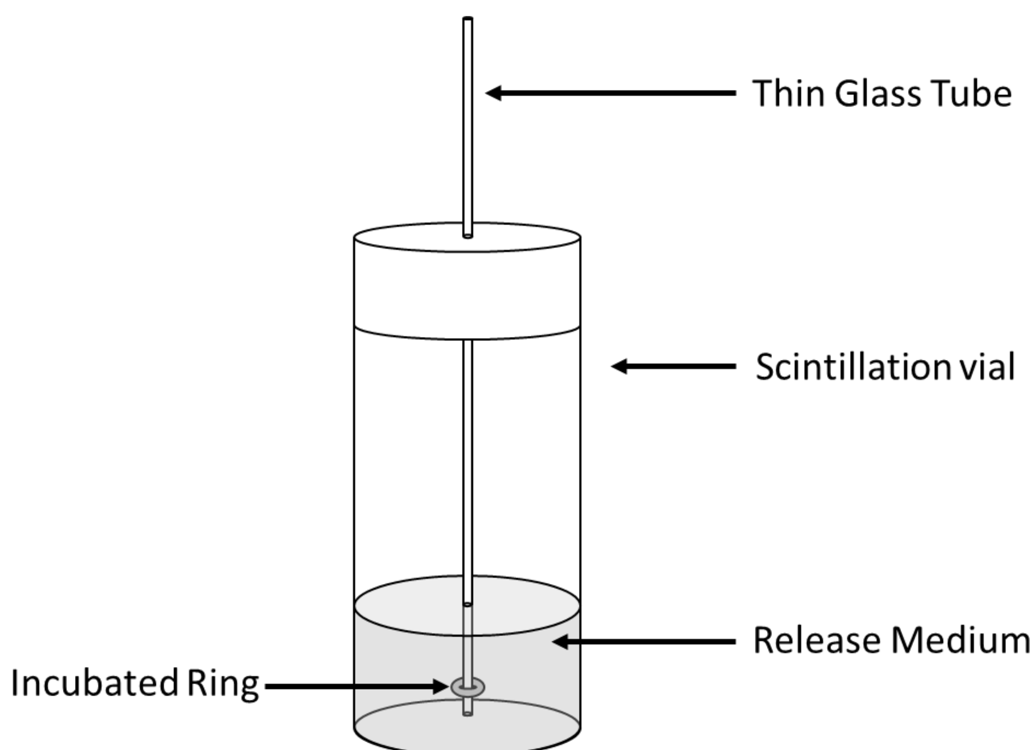


Figure 2.2. The design used for testing the release of the rings. The rings were hung in scintillation vials containing 3 ml of the release medium to maintain sink conditions using thin glass tubes to avoid floating

The rings were incubated individually in 50% isopropanol/water before setting them in an orbital shaker at 60 RPM, 37°C. The medium was replaced every 24 Hrs for 5 days, and then every 48 Hrs for the rest of the experiment. HPLC analyses were performed immediately using the method mentioned earlier. The cumulative release

was calculated by integrating the area under the curve in the release graph using LabSolutions Analysis Data System (Shimadzu). Triplicate samples were analysed.

2.8. Animal resources, conditions and ethical approval

2.8.1. Intra-Vaginal Administration of the Rings

All protocols and procedures were approved by the Animal Ethics Committee (GU Ref No: MSC/06/16/AEC) prior to commencing animal work.

Intra-vaginal silicone rings (approximately 0.75 mm thickness and 4.2 mm in diameter) loaded with 1% Alisertib (MLN8237) or drug-free were manufactured using the method mentioned earlier.

Clinical safety studies were carried out on FVB mice. At the onset of treatment, the mice were approximately 8-12 weeks. The mice were divided into 3 groups of 5 mice each. The first group was not treated. The second group, received drug-free rings as a placebo treatment. The last group, received drug-loaded rings (table 2.5).

Table 2.5. Randomization of the mice into the three test groups.

Group	Number in group	Treatment
1	5	Control (No treatment)
2	5	Placebo (Drug-free rings)
3	5	Treatment (Drug-loaded rings)

The weight of each mouse was recorded before implanting the rings and then every 24 hours for 7 days. The vaginal rings were lubricated with K-YR Brand Jelly, and introduced into the vaginas of mice in the test and placebo groups using curved tweezers.

On day 7, the mice were euthanized via inhalation of carbon dioxide. Genital organs were collected for analysis.

2.8.2. Tissue processing and sectioning

Genital organs were fixed in 10% neutral buffered formalin (3.7% Formaldehyde, 6.5 g/l Na_2HPO_4 and 4 g/l NaH_2PO_4 in distilled water) for 3 days before moving them to 70% ethanol for another 3 days. Tissues were processed in a Shandon Excelsior ES1 Tissue Processor (Thermo Scientific) as outlined in table 2.6.

Table 2.6. Steps of tissue processing in Shandon Excelsior ES Tissue Processor (Thermo Scientific)

Step	Reagent	Process	Time	Temp	Vacuum(Va/P)
1	70% Ethanol	Dehydration	30 min	37°C	No
2	90% Ethanol	Dehydration	30 min	37°C	No
3	95% Ethanol	Dehydration	30 min	37°C	No
4	100% Ethanol	Dehydration	30 min	37°C	No
5	100% Ethanol	Dehydration	30 min	37°C	No
6	100% Ethanol	Dehydration	30 min	37°C	No
7	100% Xylene	Clearing / Transition	40 min	37°C	No
8	100% Xylene	Clearing / Transition	40 min	37°C	No
9	100% Xylene	Clearing / Transition	40 min	37°C	No
10	Paraffin wax	Infiltration	60 min	60°C	No
11	Paraffin wax	Infiltration	60 min	60°C	No
12	Paraffin wax	Infiltration	60 min	60°C	Yes

Tissues were then embedded in wax and 5 nM longitudinal sections were prepared using an automated rotary microtome (Leica RM2265).

2.8.3. H&E Staining

Table 2.7 shows the steps performed for staining the tissues, in addition to the reagents used.

Table 2.7. Steps of H&E staining

Process	Reagent	Vendor	Duration
Deparaffinising	Xylene	Chem-supply	3 minutes
	Xylene		3 minutes
Rehydration	Ethanol 100%		1 minute
	Ethanol 100%		1 minute
	Ethanol 95%		1 minute
	Ethanol 95%		1 minute
	Running tap water		1 minute
Nuclear Staining	Harris haematoxylin	Amber Scientific	7 minutes
	Running tap water		1 minute
Differentiation	0.5% hydrochloric acid alcohol	Amber Scientific	12 seconds
	Running tap water		30 seconds
Bluing	Scott's blueing solution	POCD Scientific	1 minute
	Running tap water		1 minute
Cytoplasmic Staining	Eosin working solution	Amber Scientific	20 seconds
	Running tap water		30 seconds
Dehydration	Ethanol 95%	Chem-supply	1 minute
	Ethanol 95%		1 minute
	Ethanol 100%		1 minute
	Ethanol 100%		1 minute
	Xylene		3 minutes
	Xylene		3 minutes

2.9. Statistical analysis

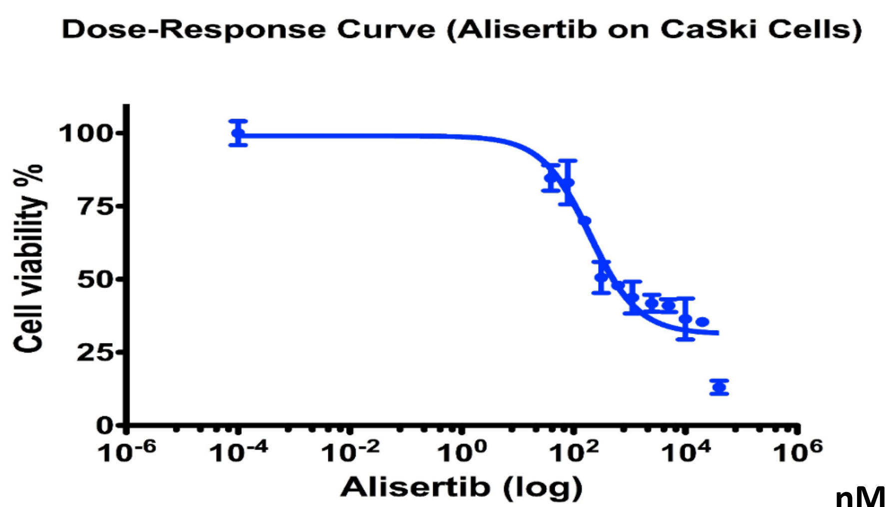
Statistical analyses were performed using IBM SPSS Statistics 22 Software and graphs were generated using GraphPad Prism™ Ver. 6 software. Error bars in relevant figures reflect the standard deviation of the mean (±SEM) observed values, and statistical analyses on data are as described in each figure. Unpaired t-test or ANOVA test where performed where relevant, and a 95% confidence interval (P-value <0.05) was used to compare controls with treated samples.

Chapter 3: Results

3.1. Setting the initial parameters for the Alisertib loaded rings

3.1.1. Determining the IC50 of Alisertib on CaSki cell line

In order to develop a vaginal delivery system for Alisertib in the vaginal setting it was important to first determine the half maximal inhibitory concentration (IC_{50}) of the drug on a cervical cancer cell line (figure 3.1). This step was crucial as a starting point to determine the target range of Alisertib release from the rings that is able to kill cervical cancer cells.



	Viability
log(inhibitor) vs. response (three parameters)	
Best-fit values	
Bottom	31.29
Top	99.08
LogIC50	2.290
IC50 (nM)	194.9
Span	67.78
Std. Error	
Bottom	2.580
Top	4.631
LogIC50	0.1183
Span	4.897
95% Confidence Intervals	

Figure 3.1. IC50 of Alisertib on CaSki cell Line. CaSki cells were treated with 11 concentrations of Alisertib ranging from 40 mM to 40 nM over 72 Hr. MTT cell viability assay was used to compare treated cells to untreated ones (represented by point on far left of graph), and absorbance data was used to generate the dose-survival curve. IC50 was calculated using GraphPad Prism Software.

Eleven concentrations of Alisertib, from 40 nM to 40 mM, were tested on CaSki cells (HPV16 positive cervical cancer cells) in triplicate, and viability was compared to untreated cells at 72 hours using an MTT assay. Using this data, dose-survival curves were generated and IC50 was calculated. It can be seen that the IC50 of Alisertib on CaSki cells was 194.9 nM (Fig 3.1).

3.1.2. Testing the drug activity after exposure to vaginal fluids

The next consideration after determining the IC_{50} was to test whether Alisertib would retain its activity in the vaginal setting, which is typically hydrophilic with a low pH (4.2). To test this, 25 mM of Alisertib/DMSO solution was diluted into Simulated Vaginal Fluid (SVF) to a final 1X concentration and left to incubate for 1 to 3 hours. The solutions were then diluted into tissue culture media to a final concentration of 200 nM. Alisertib not exposed to SVF and untreated cells were used as positive and negative controls respectively. After 72 hours, an MTT assay was undertaken and cell-viability compared (figure 3.2).

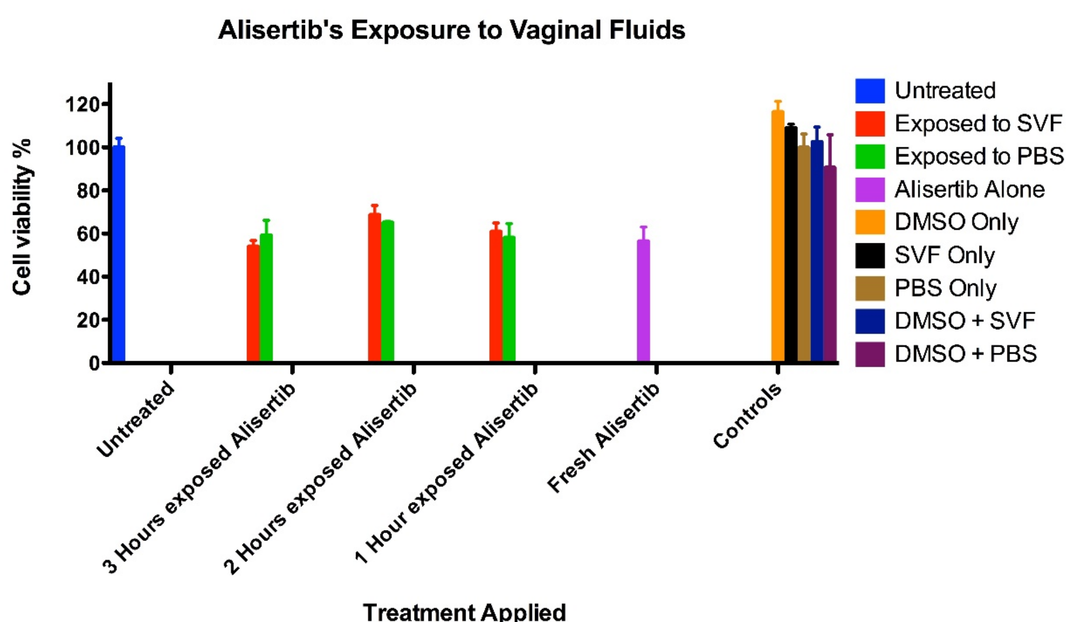


Figure 3.2. Alisertib retained activity upon exposure to vaginal fluids. CaSki cells were treated with Alisertib exposed to either Simulated Vaginal Fluid (SVF) or phosphate buffered

saline (PBS). Cells treated with fresh unexposed Alisertib and untreated cells were used as controls. MTT cell viability assay was used to compare cell viability of the groups. ANOVA test was performed and Tukey post-hoc was used to compare means of the groups.

According to ANOVA analysis (table 3.1), fresh Alisertib had no statistical difference in efficacy compared to samples exposed to SVF for 3 hours (mean difference= 2.38, 95% CI: -31.0259, 35.7845, $P > 0.05$). This result suggests that Alisertib would still be active in the vaginal setting up to 3 hours post-exposure.

Table 3.1. ANOVA test shows no significant difference of Alisertib activity after being exposed to simulated vaginal fluids

Fresh Alisertib (A) Mean (SD)	SVF 3 (B) Mean (SD)	PBS 3 (C) Mean (SD)	SVF 2 (D) Mean (SD)	PBS 2 (E) Mean (SD)	SVF 1 (F) Mean (SD)	PBS 1 (G) Mean (SD)	F
56.38 (11.55)	54 (4.75)	59.03 (12.25)	68.95 (7.61)	65.03 (1.04)	60.86 (6.96)	58.07 (11.41)	0.99
* significant at $p < 0.05$, ** significant at $p<0.01$, ***significant at $p<0.001$.							

3.1.3. Alisertib bioactivity after exposure to elevated temperature (Thermostability of Alisertib)

Despite the fact that a low IC_{50} and stability upon exposure to simulated vaginal fluids are key factors in considering whether a drug can be locally delivered in the vaginal tract, it is also necessary to ensure that the drug will survive the manufacturing process when embedded into silicone-based vaginal rings as this process involves exposure to elevated temperature.

To address this, the biological stability of Alisertib when exposed to 80°C for 30 minutes was investigated, and its biological activity was determined by treating of CaSki cells for 72 hours followed by an MTT assay. Comparison was made to the same concentration of unheated Alisertib (figure 3.3).

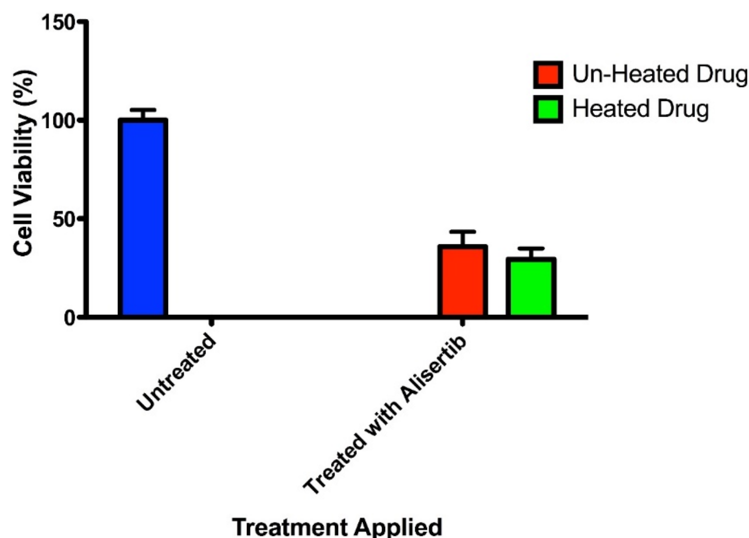


Figure 3.3. Alisertib retained biological activity after exposure to elevated temperature. CaSki cells were treated with 200 nM Alisertib either without heating or heated to 80°C for 30 minutes. MTT cell viability assay was used to compare cell viability of the groups after 72 Hrs of incubation. T-test was performed to compare means of the groups.

It can be seen that there was no difference in the efficacy of heated and non-heated Alisertib in reducing cell viability of CaSki cells ($p > 0.05$) (table 3.2).

Table 3.2. T-test shows no statistical significant difference in Alisertib activity after being exposed to elevated temperature

Variable	Unheated Alisertib Mean(SD)	Heated Alisertib Mean(SD)	T	P value
Cell Viability	35.85 (13.02)	29.43 (9.47)	0.69	> 0.05

3.2. Developing a quantitative-HPLC method for Alisertib

As Alisertib was a suitable candidate for incorporation into silicone-based intra-vaginal rings for topical delivery in the vaginal tract, development of a reliable method of Alisertib quantification was needed, so the release profile of the drug from these rings could be studied. To do this, a reverse phase high pressure liquid chromatography (HPLC) method was developed and validated.

3.2.1. Identifying Lambda Max of Alisertib by studying the absorbance spectrum

HPLC readings are generally acquired by absorbance of the light signal as detected by the photodiode array detector (PDA). This detector measures absorption values after separation through the HPLC column and this allows the calculation of drug concentration. Thus, lambda max (λ_{max}) of Alisertib was examined by scanning a solution of Alisertib over a wave length of 200-650 nm on the Multiskan^{TM} GO microplate spectrophotometer with the solvent used as a control. Lambda max of Alisertib was identified at 314-316 nm (figure 3.4). Therefore, PDA acquisition was set to include this range in the HPLC method.

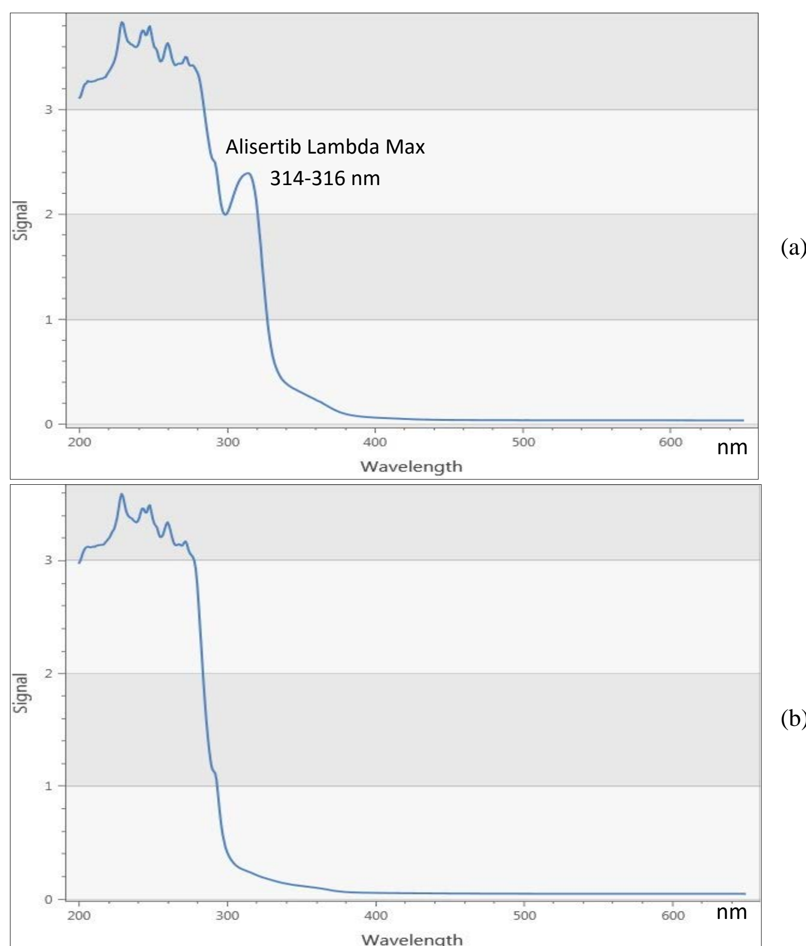


Figure 3.4. Absorbance over 200-650 nM identified Lambda max of Alisertib 314-316 nM (a) A 1 mg/ml solution of Alisertib in 1% Formic Acid/CAN with a peak at 314-316 nM (b) Blank (1% Formic Acid/ACN).

3.2.2. HPLC method development

Alisertib is a highly hydrophobic compound (logP=5.74, as predicted using MarvinSketch Software, ChemAxon). The initial method of HPLC separation aimed to elute any impurities that are soluble in the solution while Alisertib is retained and attached to the fatty chains of the C18 column. This was followed by a gradual increase of the organic mobile phase concentration to allow the compound to travel through the column and be eluted over time (figure 3.5). Initially, pure samples of Alisertib dissolved in 1% Formic Acid/ACN and diluted in 0.1% Formic Acid/ACN were injected through the HPLC system.

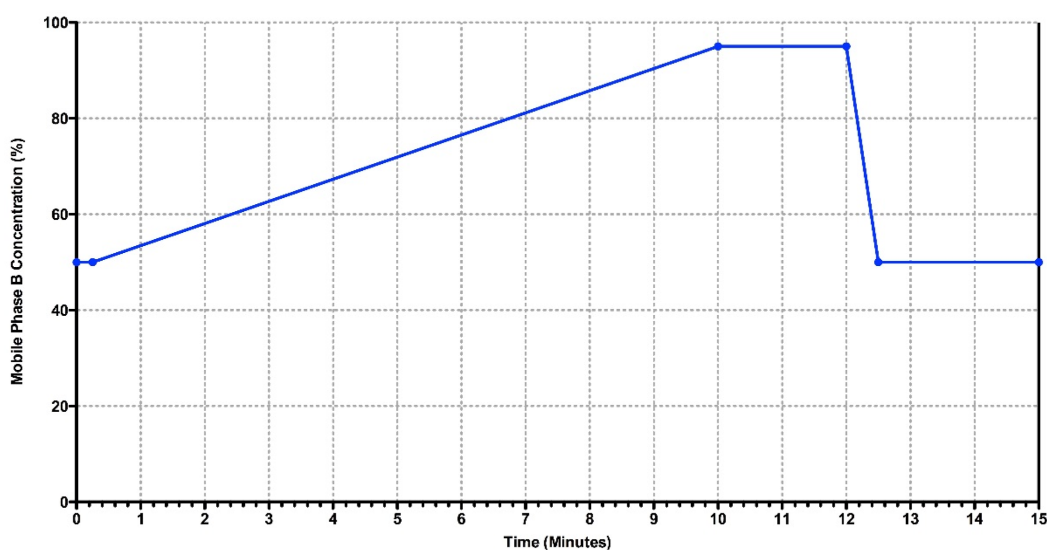
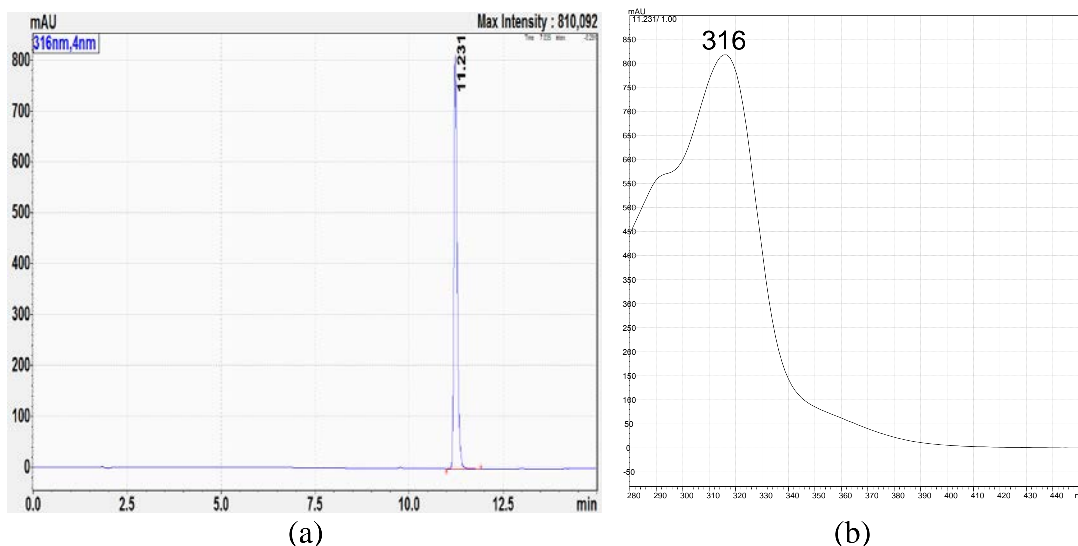


Figure 3.5. Initial HPLC Run Conditions. Gradual increase of the organic mobile phase over 10 minutes reaching 95% at minute 10.

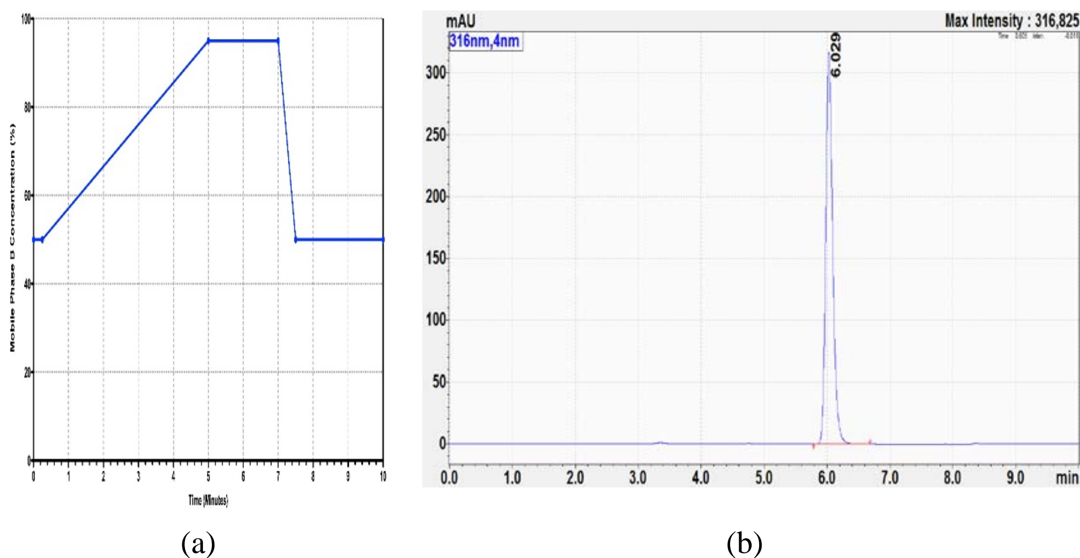
Retention time was 11.23 minutes and λ_{max} was confirmed at 316 nm (figure 3.6.). It is clear from figure 3.6.a. that the gradient could be accelerated to get shorter retention time and allow the column to equilibrate properly after the drug was eluted and the gradient restored to the initial parameter (50% mobile phase B).



(a) (b)

Figure 3.6. Typical chromatogram of the initial HPLC run. (a) Alisertib standard injected into the initial method eluted at 11.231 minutes with 15 minutes total run time (b) absorbance spectra of the initial run with a peak in absorption at 316nm.

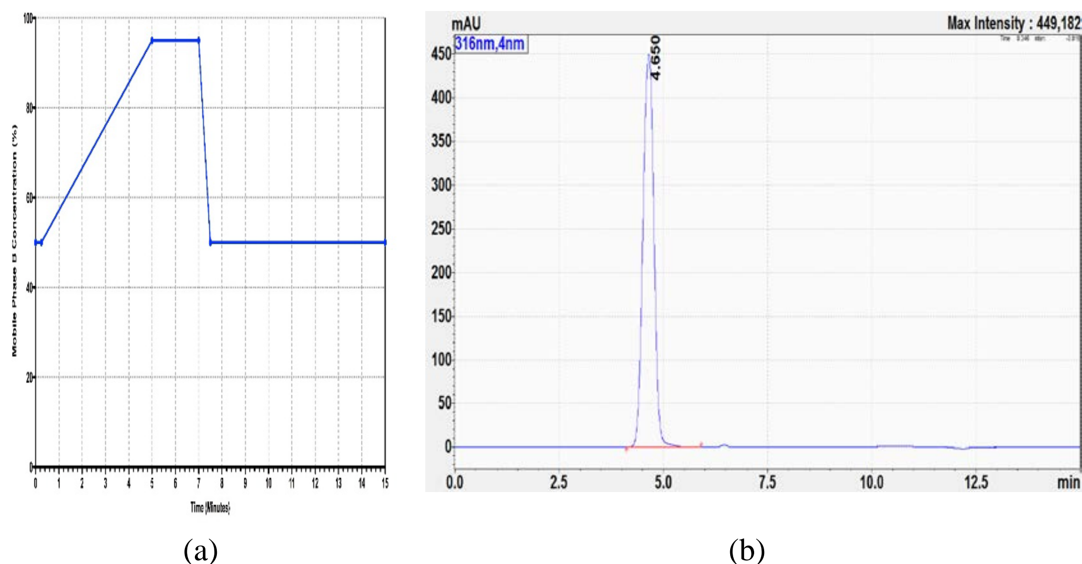
In order to achieve shorter retention time of the compound, the gradient of mobile phase B over time was accelerated (figure 3.7.a). This resulted in a decreased retention time (6.029) with a total run time of 10 minutes (figure 3.7.b).



(a) (b)

Figure 3.7. Typical chromatogram of the tweaked HPLC run. (a) Gradual increase of the organic mobile phase over 5 minutes reaching 95% at minute 5 with 10 minutes total run time (b) Alisertib standard injected into the tweaked method eluted at 6.029 minutes.

The gradient and the run time were adjusted many times in order to allow the column to equilibrate properly while maintaining a reasonable processing time (4 samples per hour). The final gradient conditions are described in figure 3.8. Retention time was changed slightly with each replacement of the guard column.



(a) (b)

Figure 3.8. Typical chromatogram of the final HPLC run. (a) Gradual increase of the organic mobile phase over 5 minutes reaching 95% at minute 5 with 15 minutes total run time (b) Alisertib standard injected into the final method eluted at 4.65 minutes.

3.2.3. Generating a calibration curve for quantifying the compound in solution.

The final HPLC method achieved a reasonable processing time of the samples and allowed the column to be equilibrated properly between runs. However, in order to move forward, the generation of a calibration curve of standard concentrations was needed to allow the determination of the Alisertib concentration in unknown samples.

To generate a calibration curve, a stock solution of Alisertib (1 mg/mL) was prepared in 1% Formic Acid/Acetonitrile. The stock solution was diluted in 0.1% Formic Acid/Acetonitrile to prepare the external standard concentrations. Six concentrations were injected through the HPLC in triplicate. The concentrations used were 2 μg/ml, 500 ng/ml, 200 ng/ml, 100 ng/ml, 50 ng/ml and 20 ng/ml. These

reflected molarities of 3854.2 nM, 963.54 nM, 385.43 nM, 192.71 nM, 96.35 nM and 38.54 nM respectively (MW=518.92).

In order to improve the peaks, it was necessary to mix all samples with the organic mobile phase (Acetonitrile) before injecting into the HPLC machine. One part of each standard solution was mixed with 2 parts of Acetonitrile and vortexed before HPLC measurements. The standard solutions were stored in -80°C and a new calibration curve was made using the same standard concentrations each time the mobile phase solutions were refilled or the guard column was replaced.

The average area-under-the-curve was calculated from triplicate measurements using LabSolutions Analysis Data System (Shimadzu) and used to generate a linear standard curve intercepting (0,0) (Figure 3.9). Linear regression analysis was used to generate a line of best fit, which had an R^2 value of 0.9999, suggesting an excellent fit.

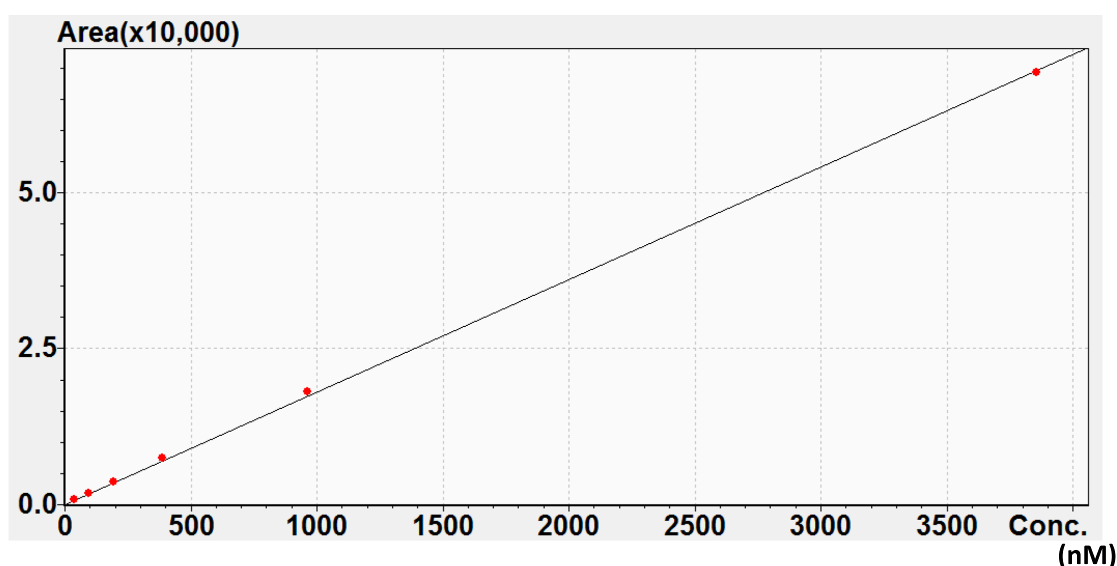


Figure 3.9. Final Calibration curve for Alisertib. Generated using standard concentrations (3854.2 nM, 963.54 nM, 385.43 nM, 192.71 nM, 96.35 nM and 38.54 nM). The mean of the area under the peaks curves were used to generate a linear type standard curve intercepting (0,0) where R^2 value was 0.9998571. Limit of Quantification (LOQ) = 143.99 nM, and Limit of Detection (LOQ) = 47.52 nM.

3.3. Extracting Alisertib from SVF solutions

Studying the release of the rings in simulated vaginal fluids (SVF) would provide a better understanding of the release profile of the drug from silicone devices. Since Alisertib is a hydrophobic weak acid/base molecule, it was important to validate the efficacy of the developed HPLC in measuring the concentration of the drug in acidic and hydrophilic medium as SVF. To achieve this, firstly the effect of the SVF on the extraction method was investigated, followed by examining the measurement in pure SVF.

3.3.1. The effect of SVF on extraction efficacy

This test was undertaken to examine the efficiency of the developed HPLC method in extracting and quantifying Alisertib from its solutions after being exposed to SVF. A stock solution of Alisertib (1 mg/mL) was prepared in 1% Formic Acid/Acetonitrile. The stock solution was diluted in 0.1% Formic Acid/Acetonitrile to prepare a series of concentrations as shown in figure 3.10.a. The standard solutions were then spiked in SVF as 1 in 10 to prepare the SVF samples (figure 3.10.b). Finally, the method mentioned above to precipitate proteins was performed for all solutions before they were injected through the HPLC system to determine the extraction efficiency.

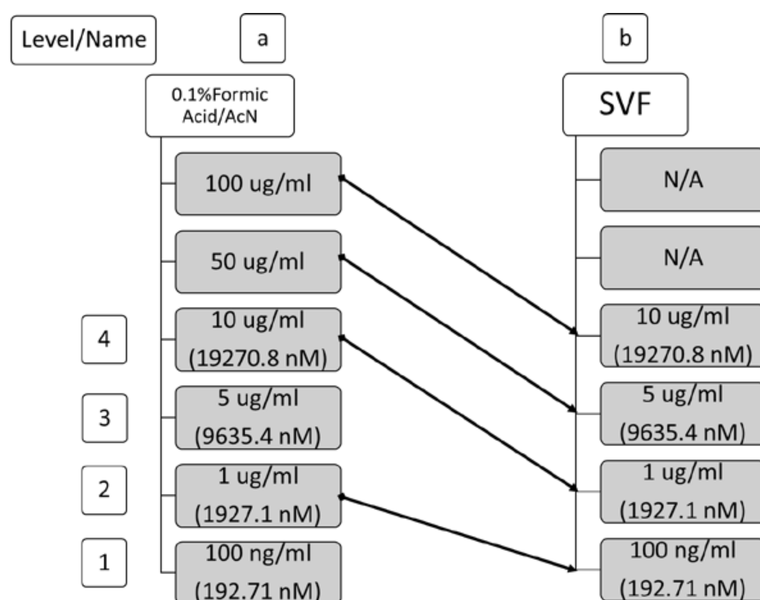


Figure 3.10. Sample preparation for studying the effect of SVF on the extraction efficiency. (a) Prepared by diluting a stock solution of Alisertib (1 mg/ml Alisertib in 1% Formic Acid in Acetonitrile) in 0.1% Formic Acid/Acetonitrile (b) prepared by mixing 1 part of solutions in column a with 9 parts of SVF.

Four concentrations of Alisertib (19270.8 nM, 9635.4 nM, 1927.1 nM and 192.71 nM) were measured and compared between the two solvents (figure 3.11). One way-ANOVA and Tukey post-hoc tests show no significant difference between groups at lower concentrations (192.71 nM and 1927.1 nM). However, significant differences in extraction efficiency were observed at higher concentrations.

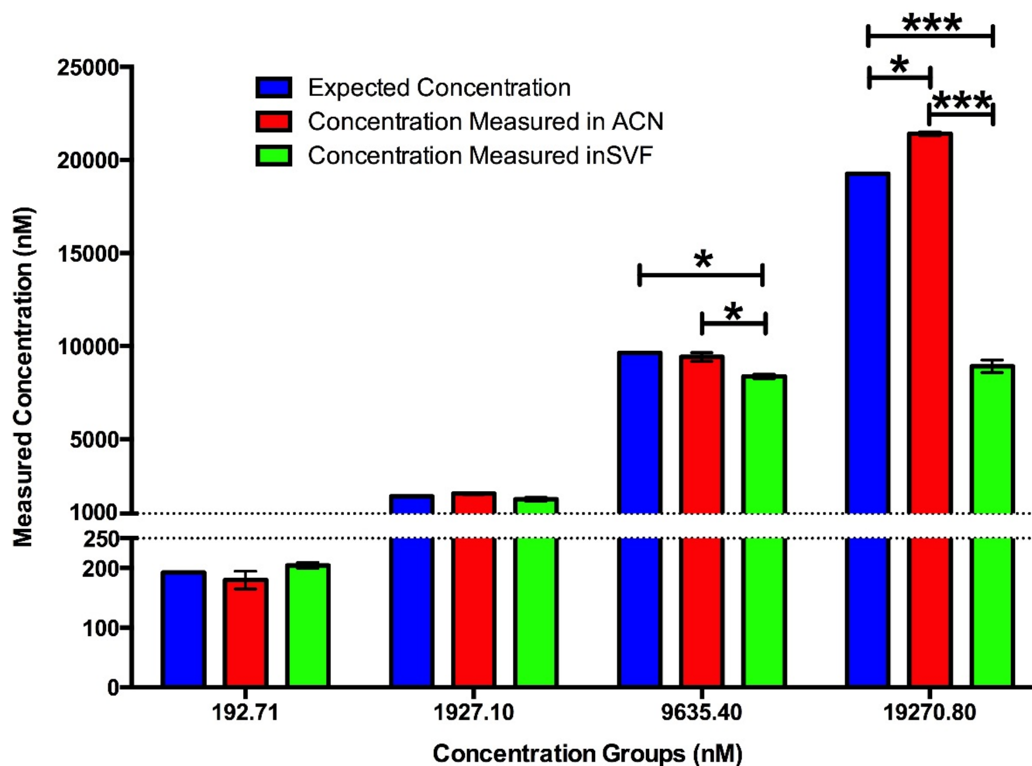


Figure 3.11. Extraction efficacy after diluting standard solutions in SVF as 1 in 10 is affected at high concentrations. Solutions were prepared as in figure 3.10 before triplicates were compared between standard solutions and mixed with SVF solutions (*:p<0.05, **:p<0.01, *** p<0.001)

The significant drop in concentration measurement at high concentrations suggests over-saturation of the compound around 5 µg/ml. All further tests on SVF solutions were therefore performed on concentrations lower than 1927.1 nM.

3.3.2. Extraction Stability of Alisertib in SVF Solutions

The efficacy of the extraction method was then investigated by quantifying Alisertib concentrations dissolved in pure SVF. Achieving reliable consistency in measurements over time is essential to study the release profile of the silicone rings in SVF as a release medium. This test was performed by dissolving random amounts of Alisertib in SVF and immediately measuring the concentration using the developed HPLC method. The remaining solution was kept incubated at 37°C for 24 hours and sampled at 3, 6, 12, and 24 hours (figure 3.12).

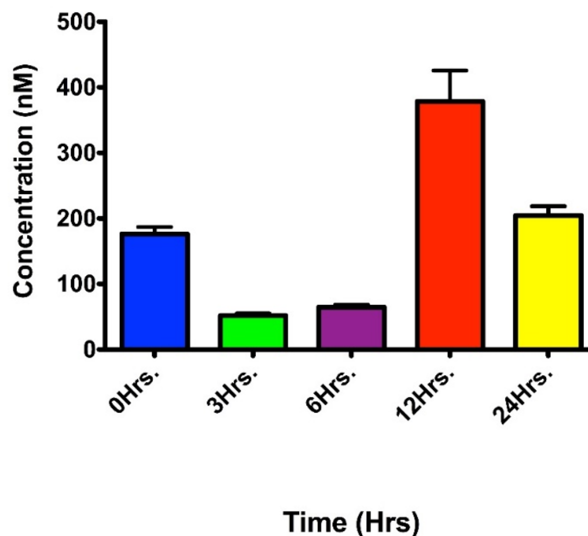


Figure 3.12. An example of Alisertib dissolved in SVF showing inconsistent HPLC readings over time. The same sample of Alisertib dissolved in SVF was measured using HPLC at five different time points (0, 3, 6, 12 and 24 Hrs.). The sample was kept at 37°C at all times during the test duration. Inconsistency in measurement over time was noticed.

HPLC data shows inconsistency in measuring Alisertib concentration over time in SVF. The initial concentration reading of 176 nM dropped sharply in 3 hours to around 52 nM before it reached the highest peak in 12 hours at around 378 nM (higher than the initial concentration).

The random pattern of measured concentrations over time suggests a drawback in the extraction efficiency from pure SVF. The fact that Alisertib retained its anti-cancer activity upon exposure to SVF (section 3.1.2), in addition to the random HPLC recovery amounts, excludes the possibility of the compound's degradation in SVF. To further investigate this, more tests were performed.

3.3.3. Altering the concentration of ACN in the samples

The initial method was set to dilute all samples at a ratio of 1:2 in acetonitrile (ACN) before running the HPLC method to precipitate proteins and improve the quantitative peak (diluting in the organic mobile phase). Here many dilutions in ACN

were tested to identify the ratio that generated the best recovery in SVF solutions. The addition of ACN is necessary to precipitate the proteins in SVF before injecting through the HPLC system. Therefore, protein-free SVF was used to allow smaller amounts of ACN to be mixed with the samples.

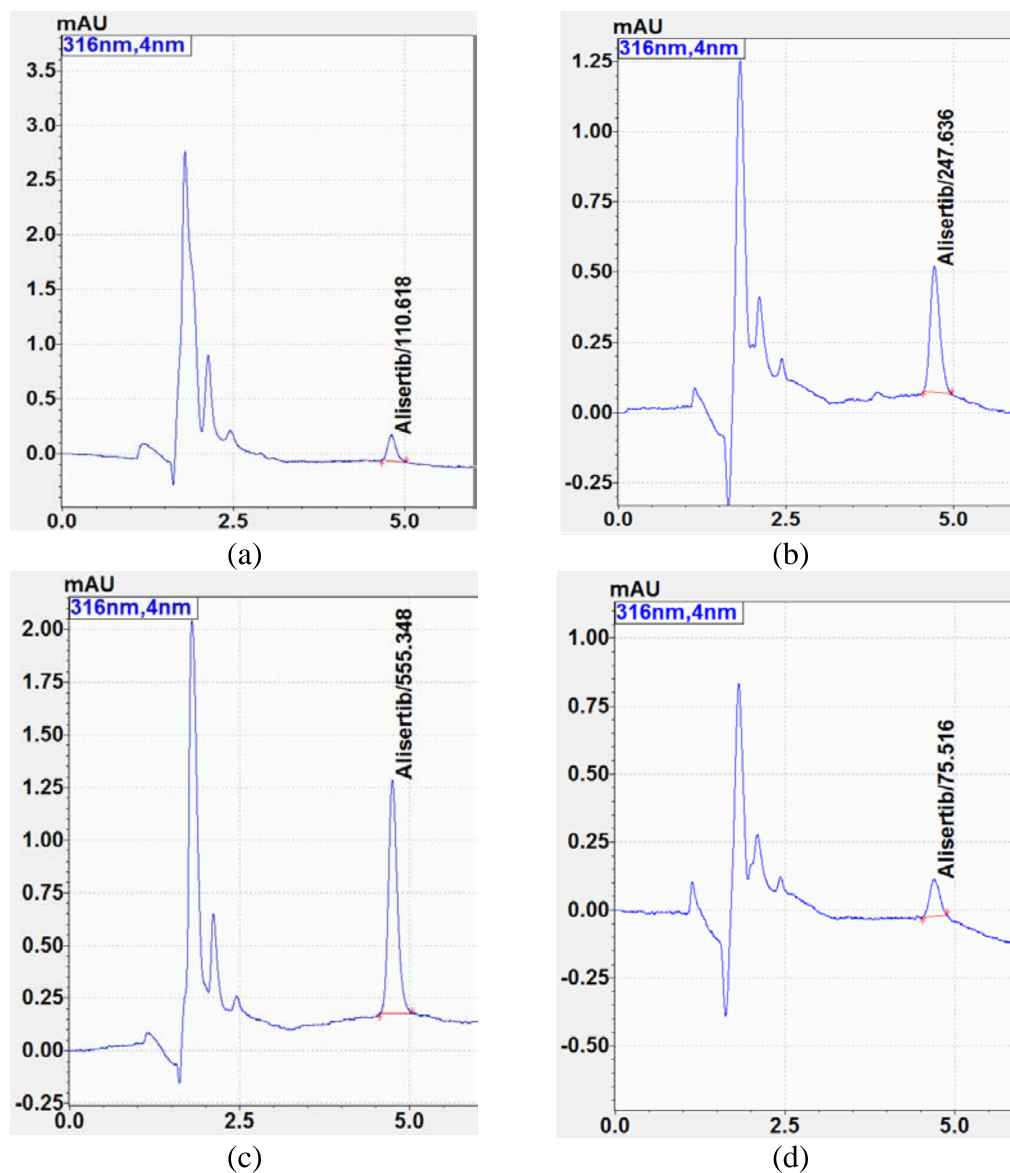


Figure 3.13. A mix ratio of 1:2 sample:ACN generated the largest peaks in SVF samples. Samples of Alisertib/SVF solutions were mixed in different ratios with ACN before measuring Alisertib concentration using the developed HPLC method. (a) 1:0 sample:ACN (b) 1:1 sample:ACN (c) 1:2 sample:ACN (d) 1:3 sample:ACN

Ratios of 1:1, 1:2 and 1:3 sample:ACN (v/v) were tested. Theoretically, smaller dilutions in ACN should generate larger peak areas (more concentrated samples).

However, the 1:2 ratio samples generated the largest peaks (figure 3.13). These findings suggest that a 1:2 ratio keeps an acceptable balance between acidity (which is explored later) and dilution of the samples.

3.3.4. Studying the effect of pH on the measurement efficacy

The ratio of mixing with ACN was found to have a significant effect on the extraction efficacy in HPLC. This may have been due to the change in pH of SVF as a result of mixing with ACN. To further investigate this theory, the pH of the final samples was raised using various volumes of 0.1M NaOH prior to measuring the concentration through the HPLC. Table 3.3 summarizes the preparation of the altered pH solutions.

Table 3.3. Samples preparation design to study the effect of pH on the efficacy of the HPLC measurement in SVF solutions

Final Sample ID	Original solution	ACN	0.1M NaOH
The original method	33.33%	66.67%	0%
A	33.33%	66%	0.67%
B	33.33%	65%	1.67%
C	33.33%	64.33%	2.33%
D	33.33%	63.33%	3.33%
E	33.33%	50%	16.67%

Figure 3.14 shows the HPLC chromatogram of the samples described in table 3.3.

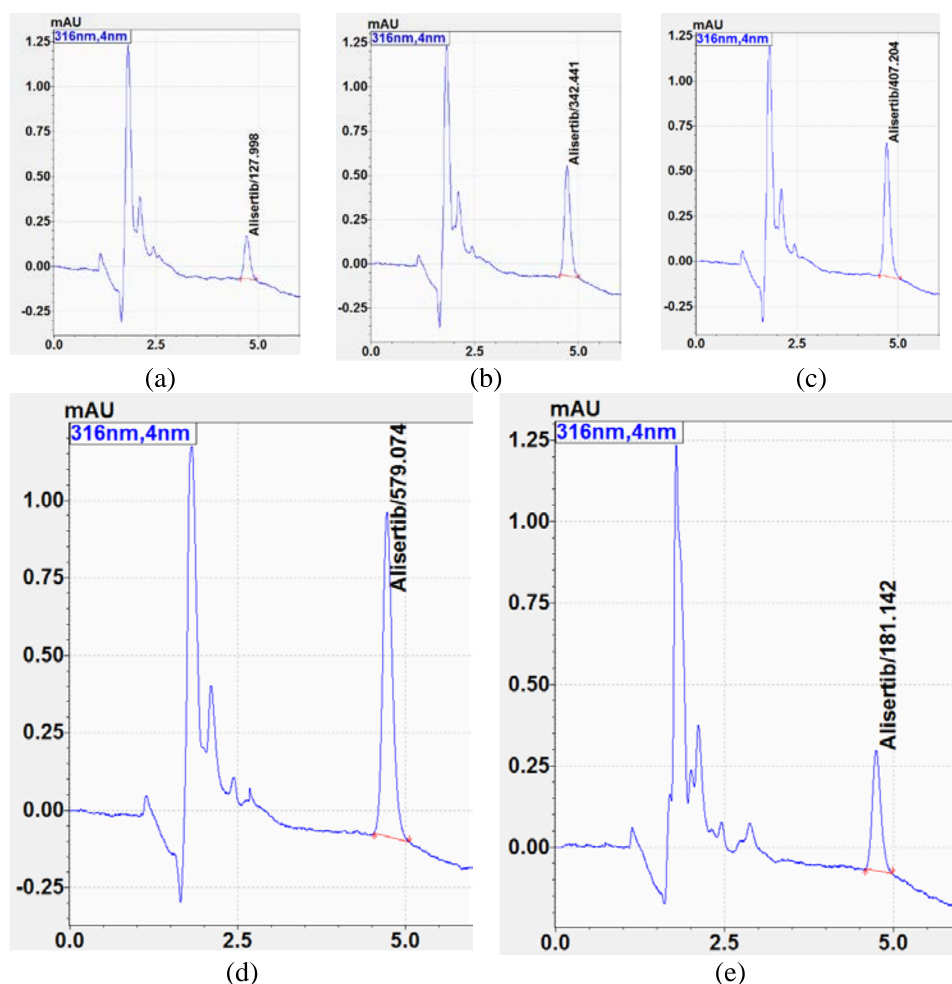


Figure 3.14. Raising the pH of the samples significantly affected the extraction efficacy of HPLC in SVF solutions. Samples of Alisertib/SVF solutions were mixed in different ratios with 0.1M NaOH before measuring Alisertib concentration using the developed HPLC method. (a) solution a in table 3.3 (b) solution b in table 3.3 (c) solution c in table 3.3 (d) solution d in table 3.3 (e) solution e in table 3.3

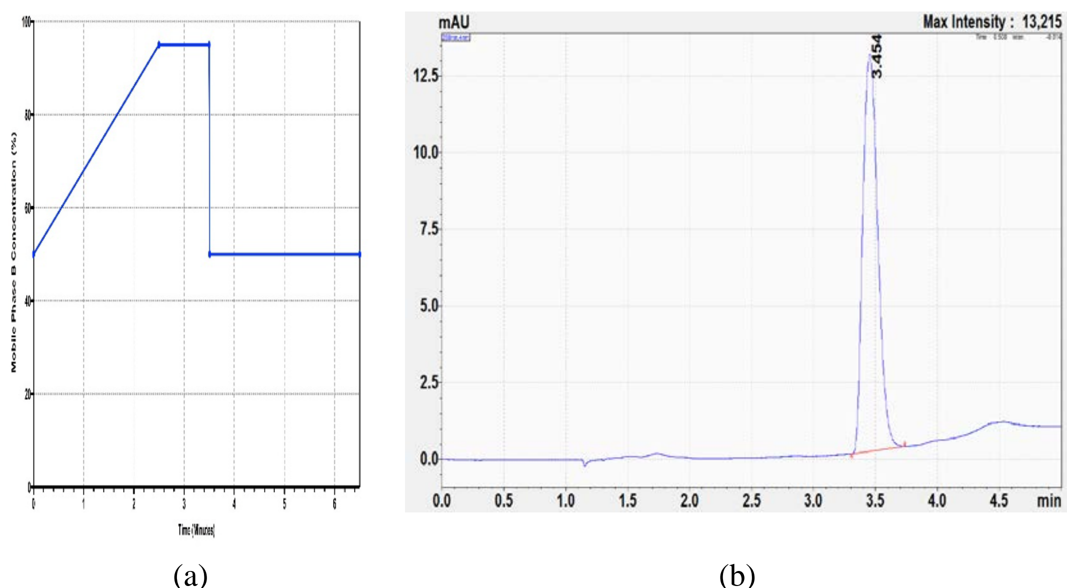
It is clear from figure 3.14 that measurement of Alisertib concentration was initially improved proportionally with the added concentration of NaOH. However, at higher concentrations of NaOH (figure 3.14.e), a drop in the measurement was recorded. Therefore, it was not possible to identify a reliable specific point as the best ratio of measurement.

3.3.5. LC-MS Analysis to explore any undetectable portion of the drug in SVF solutions

The previous data suggest the presence of an undetectable form of Alisertib through an ordinary HPLC method when dissolved in SVF. To confirm this theory, Liquid Chromatography-mass spectrometry (LC-MS) was employed to measure any undetected elution of the compound that was not measured by the original liquid chromatography method.

MS data was collected in *positive* ion mode using LCMS mode, where the masses of parent ions were detected and identified by the mass spectrometer as they elute from the liquid chromatography column.

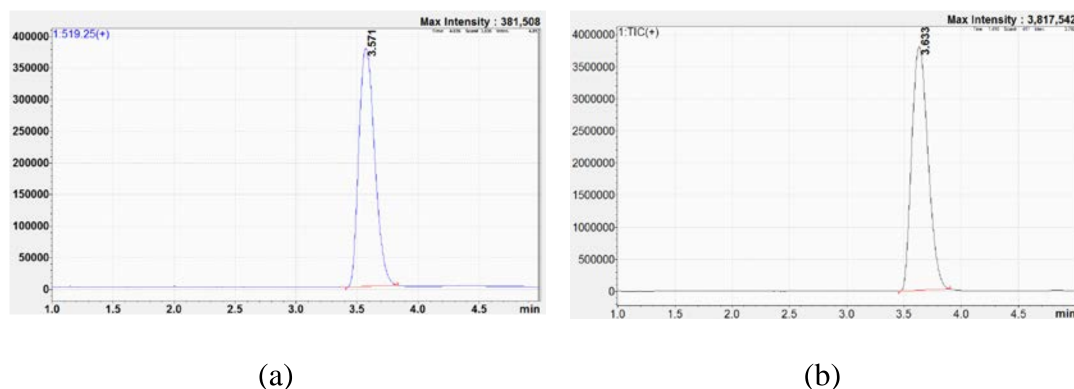
Standard samples of Alisertib dissolved in 1% Formic Acid/ACN and diluted in 0.1% Formic Acid/ACN were injected into the LCMS using the gradient shown in figure 3.15.a with a retention time of 3.454 minutes (figure 3.15.b).



(a) (b)

Figure 3.15. Typical chromatogram of the final LC-MS run. (a) Gradual increase of the organic mobile phase over 2.5 minutes reaching 95% at minute 2.5 with 6.5 minutes total run time (b) Alisertib standard injected into the final method eluted at 3.454 minutes.

Below are the results for the analysis run in MS *positive* ion mode for the Alisertib standard after identifying 519.25^{+} m/z as the parent ion of Alisertib (protonated Alisertib). Figure 3.16 shows an electron ion chromatogram (EIC) for the 519.25^{+} m/z ion after the Aliertib standard was injected (corresponding to the mass + 1 amu of Alisertib).



(a) (b)

Figure 3.16. Electron ion chromatogram (519.25 m/z) in positive ion mode for the Alisertib standard (a) Acetonitrile: The chromatogram shows one large prominent peak eluting at 3.57 minutes (b) Isopropanol: The chromatogram shows one large prominent peak eluting at 3.633 minutes

This was followed by measuring samples of Alisertib dissolved in SVF through the same method (figure 3.17). Figure 3.17 shows considerable peaks of 519.25 m/z ion at 1.349 and 1.599 minutes and no similar early peaks were detected in acetonitrile or isopropanol samples (figure 3.16.a and 3.16.b, respectively).

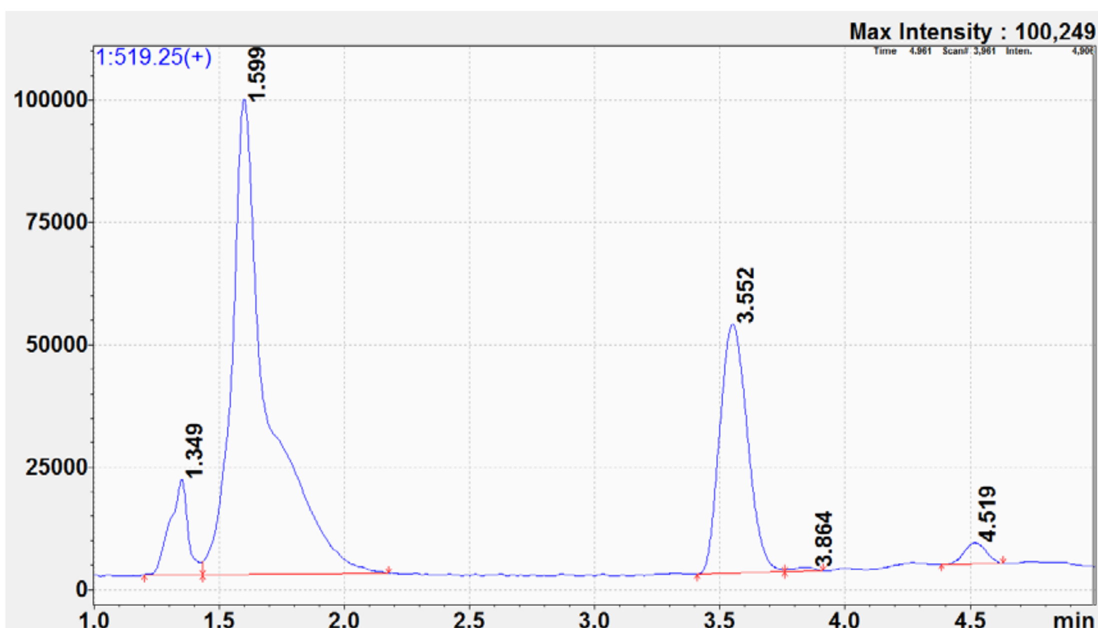


Figure 3.17. Electron ion chromatogram (519.25^{+} m/z) in positive ion mode for the Alisertib solution in SVF. Considerable peaks of 519.25 m/z ion at 1.349 and 1.599 minutes were recorded.

The results confirm the presence of an undetectable form(s) of Alisertib in SVF solutions. Thus, quantification of Alisertib in SVF was found to be unreliable using the developed HPLC method and release studies were performed in 50% isopropanol/water as a release medium. All the following studies were performed on 50% isopropanol/water only.

3.4. Measuring Alisertib solubility in 50% Isopropanol (the release medium)

Studying the release profile of silicone devices requires keeping the drug's concentration in the release medium below 10% of saturation at all times. This is referred to as maintaining the sink condition [104, 105, 116, 135]. Alisertib solubility in 50% isopropanol/water was identified as 127.81±12.56 µg/ml (246.3±24.20 µM) using the method described previously. Therefore, the release medium volume was adjusted to maintain the drug's concentration below 24.63 µM at all times.

3.5. Extraction Stability in 50% Isopropanol solutions

We investigated the efficacy of the extraction method in quantifying dissolved Alisertib in 50% isopropanol/water. Achieving reliable consistency in measurements after incubating Alisertib/isopropanol solutions at 37°C over time was essential to study the release profile of the drug embedded in silicone rings. This test was performed by dissolving random amounts of Alisertib in 50% isopropanol/water and immediately measuring the concentration using the developed HPLC method. The remaining solution was kept incubated at 37°C for 48 hours and sampled at 3, 6, 12, 24 and 48 hours (Figure 3.18).

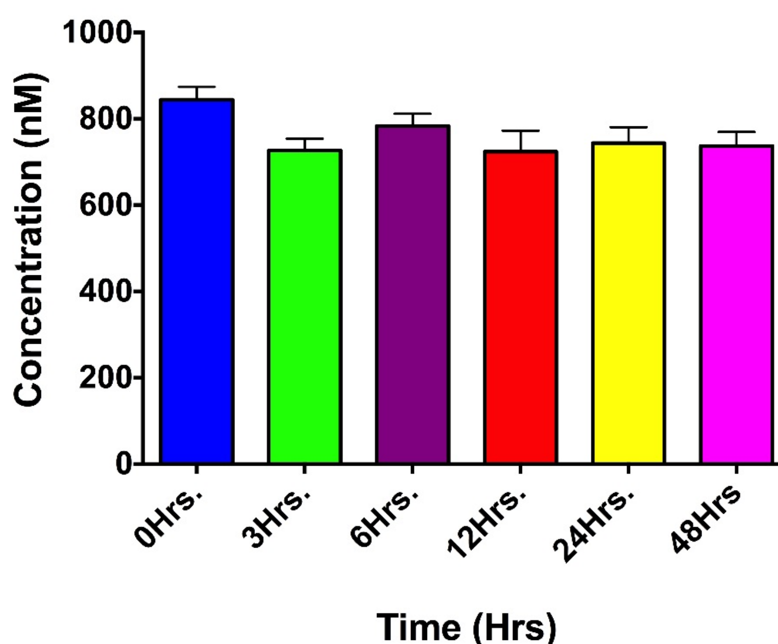


Figure 3.18. A sample of Alisertib dissolved in 50% isopropanol/water showed consistent HPLC readings over time. The same sample of Alisertib dissolved in 50% isopropanol/water was measured using HPLC at five different time points (0, 3, 6, 12, 24 and 48 Hrs.). The sample was kept at 37°C at all times during the test duration. Consistency in measurement over time was noticed in this solvent.

ANOVA test (table 3.4) showed no significant difference between groups' means. This indicates that the developed HPLC method is reliable in measuring the drug

release over 48 Hrs with no statistical significant difference in the measured concentration.

Table 3.4. ANOVA test shows no statistical significant difference in Alisertib concentration measurement over time in 50% isopropanol/water

	0 Hrs(A) Mean (SD)	**3 Hrs(B) Mean (SD)**	**6 Hrs(C) Mean (SD)**	**12Hrs(D) Mean (SD)**	**24Hrs(E) Mean (SD)**	**48Hrs(F) Mean (SD)**	**F**	**P**
Concentration nM	844.07 (52.29)	726.39 (48.01)	783.47 (48.65)	723.74 (84.62)	743.47 (63.79)	737.1 (56.56)	1.79	>0.05
* significant at p < 0.05, ** significant at p<0.01, ***significant at p<0.001.								

3.6. Ring Fabrication and release studies

From the previous set of results, it can be seen that quantifying the release of Alisertib from the silicone rings was feasible in isopropanol as a release medium. Therefore, the release from drug-loaded silicone into 50% isopropanol/water could be tested.

Initially, pilot studies on drug-loaded silicone segments (rods) with various concentrations of Alisertib were performed in order to adjust the concentration of Alisertib in the final release studies following the method in section 2.6.2. For example, the release profile of 0.69% Alisertib/silicone (w/w) rod over 11 days, where 12.149 µg of the drug was approximately released, is shown in figure 3.19.

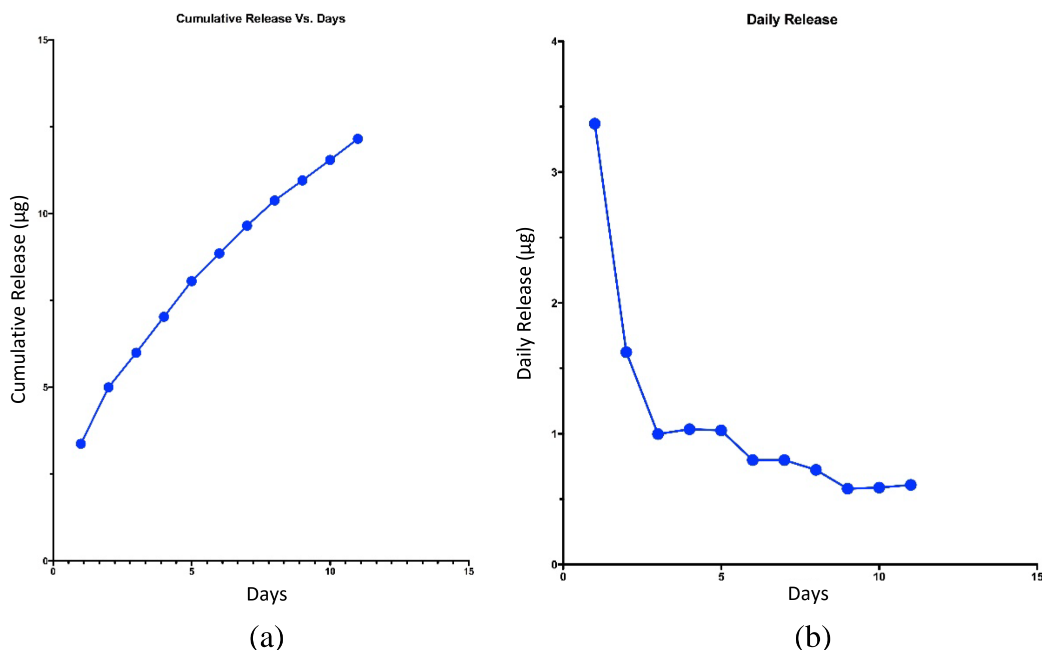


Figure 3.19. Pilot study for *in vitro* release into 50% IPA/H_2O for a silicone rod. The silicone rod weighed 26.76 mg and contained approximately 0.69% (w/w) Alisertib. Media was replaced with warmed fresh media every 24 Hrs. (a) cumulative release of drugover 11 days and (b) daily release of the drug

The next step was optimising the volume of the release medium. Triplicates of 0.5% and 1% Alisertib/Silicone (w/w) final shape rings (figure 3.20) were manufactured based on the previously described method (section 2.6.2).

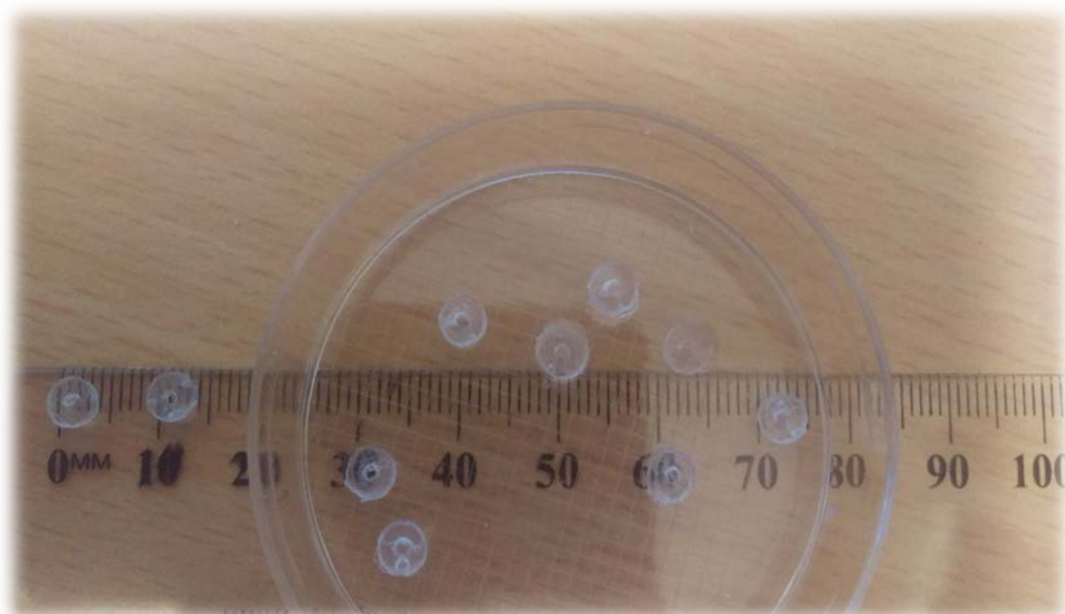


Figure 3.20. One set of the final shape mouse-sized intravaginal rings.

Three ml of release medium maintained the concentration of Alisertib below 10% of saturation (24.63μM) at all times during the period of the experiment (6 days) (figure 3.21). Thus, 3 ml of release medium was used for the final *in vitro* studies.

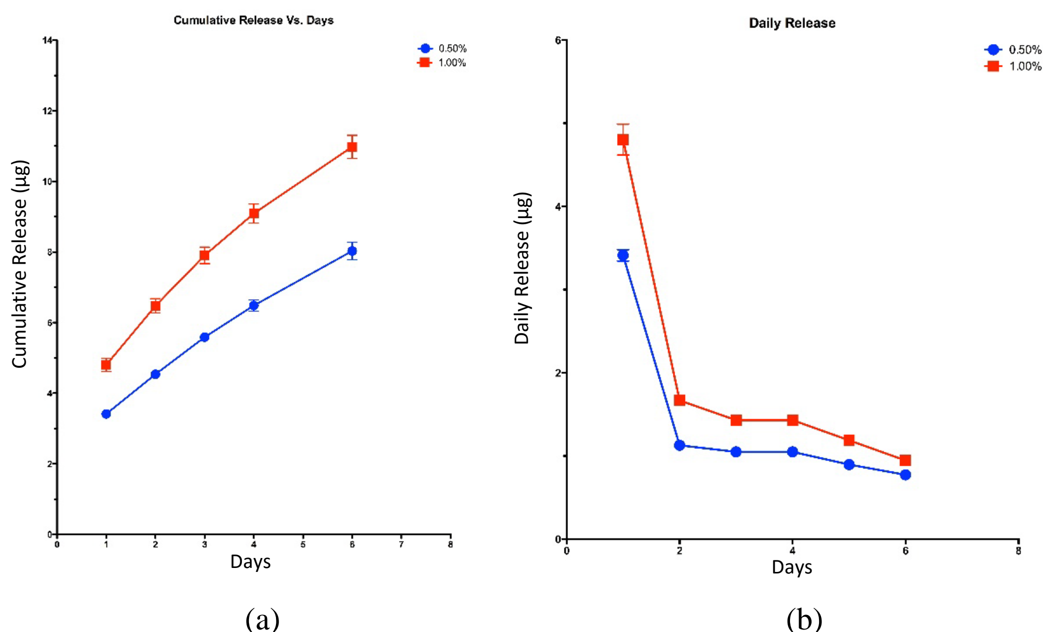


Figure 3.21. Pilot study for *in vitro* release of Alisertib into 50% IPA/H_2O for 0.5% and 1% (w/w) silicone rings. The silicone rings weights ranged from 22.65 mg to 29.01 mg. Release from 0.5% and 1% (w/w) Alisertib-loaded rings were measured over 6 days. Media was replaced with warmed fresh media every 24 Hrs. (a) cumulative release over 6 days and (b) Daily release.

It was noted that the rings contained large granules of undissolved drug. Large particle size and over-saturation may have contributed to this. A mortar and Pestle were used to grind the drug powder in the final rings before dissolving them in the silicone. This process significantly improved the texture of the rings.

Three sets of Alisertib rings were manufactured and tested in the final release experiment (containing 0.5%, 1% and 2% (w/w) Alisertib). Weights of the rings were recorded and a one-way Anova test was performed to identify any significant difference in weight between the three groups (Table 3.5). ANOVA test showed no significant difference in ring weight between the three groups ($F=1.03$, $p > 0.05$).

Table 3.5. ANOVA test showed no significant difference in weight between the rings in the three loads groups.

	0.5% (A) Mean (SD)	1% (B) Mean (SD)	2% (C) Mean (SD)	F	P
Weight mg	35.91 (0.12)	35.19 (1.36)	34.28 (2.01)	1.03	>0.05
* significant at $p < 0.05$, ** significant at $p<0.01$, ***significant at $p<0.001$.					

A daily Alisertib *in vitro* release profile from the final rings into 50% isopropanol/water over 21 days is presented in figure 3.22. It can be seen that day 1 had significantly higher released mass of the drug (the burst effect) than the remaining days. Release in day 1 ranged from 3.23 µg in the 0.5% group to 14.18 µg in the 2% group.

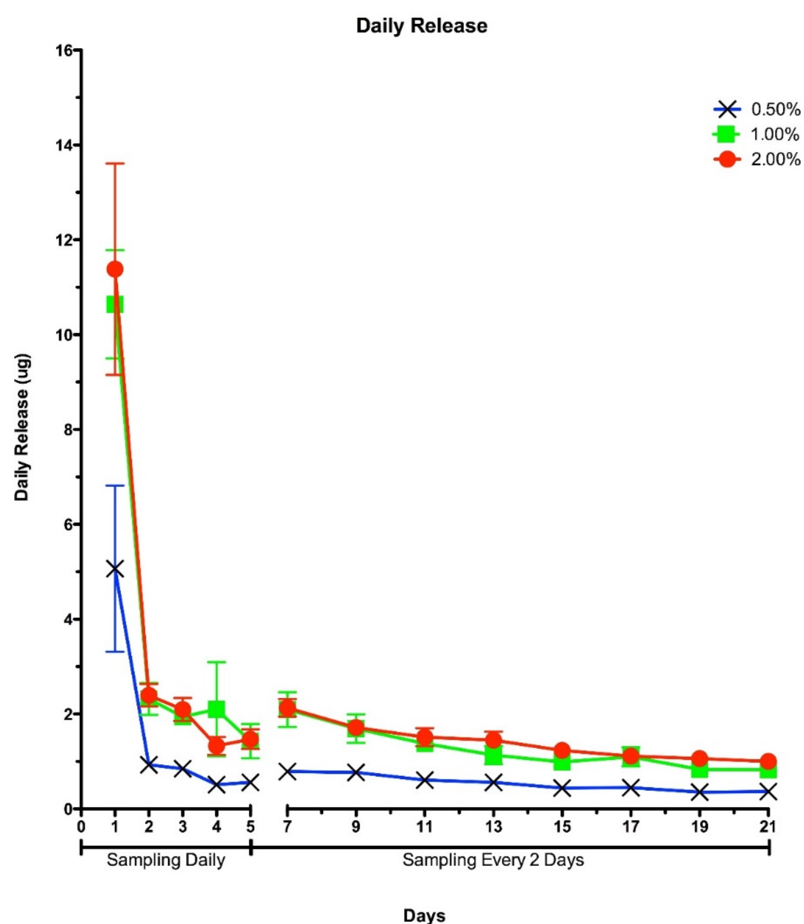


Figure 3.22. Daily release of Alisertib from the three groups of rings over 21 days. Mass of drug released was measured daily for the first five days and then once every two days for the rest of the experiment. Burst effect was noticed in the first day.

Media was replaced every 24 Hrs with fresh, warmed media for the first 5 days and release was measured immediately. For the remainder of the experiment, media was replaced every 48 Hrs. Cumulative release of the drug was calculated and plotted versus time (days) in figure 3.23. On average, approximately 6.82% of the drug load was released from the 0.5% group, 6.93% from the 1% group and 4.59% from the 2% group over the 21 days.

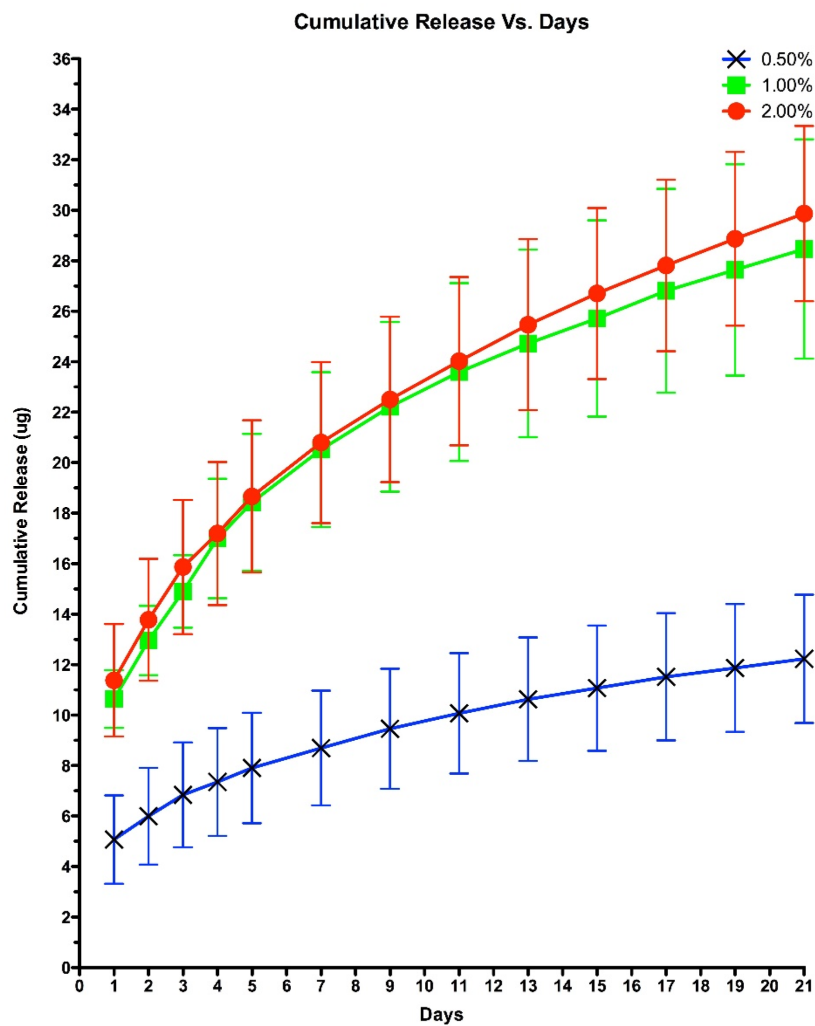


Figure 3.23. Cumulative release of Alisertib from the three groups of rings over 21 days. Cumulative mass released of Alisertib from the three groups plotted versus time (days).

The release of Alisertib into the 50% isopropanol medium obeyed root time ($t^{1/2}$) kinetics and this was confirmed by a linear cumulative release versus square-root time profile (figure 3.24).

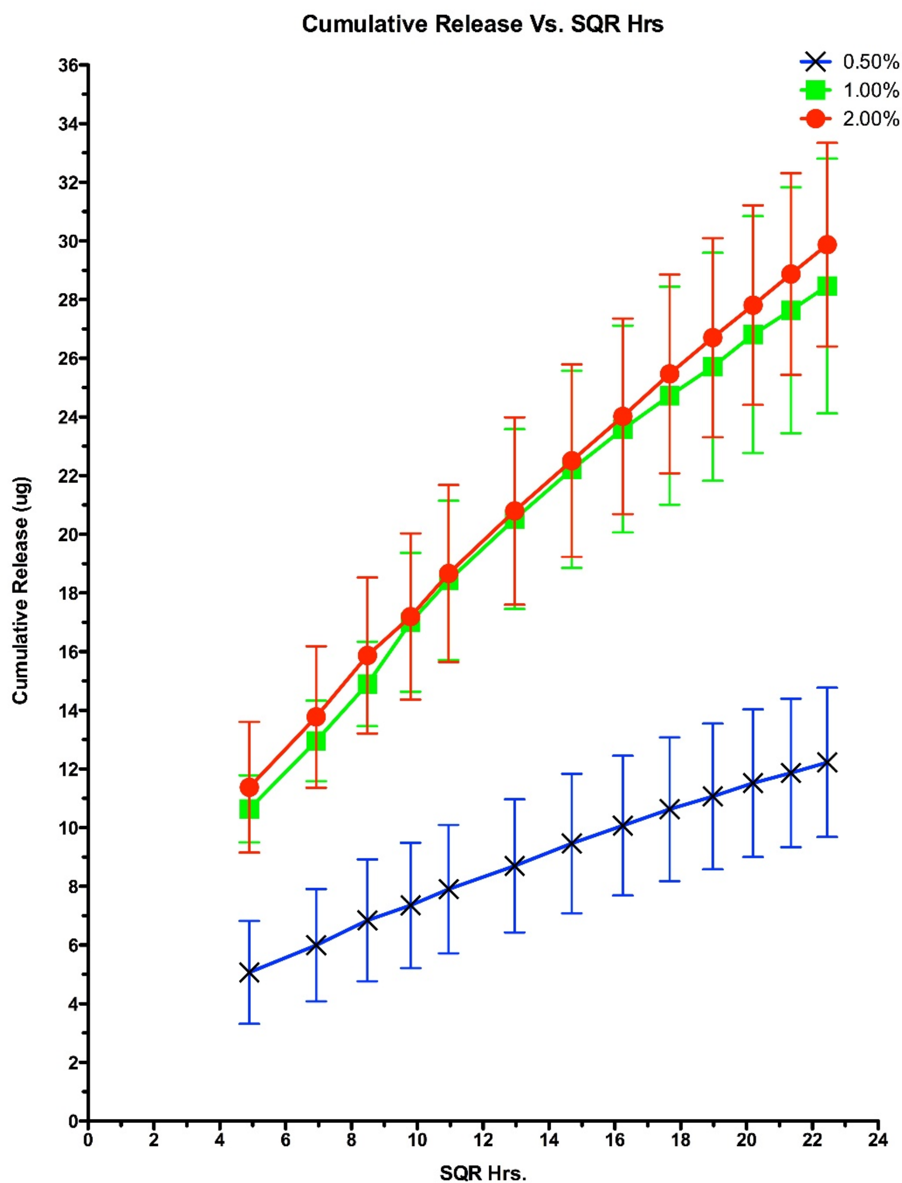


Figure 3.24. Cumulative release of Alisertib from the three groups of rings over 21 days. Cumulative mass released of Alisertib from the three groups plotted versus time (SQR Hrs).

Table 3.6 shows R^2 and slope values for each group when plotted versus $Hr^{0.5}$.

Table 3.6. R^2 and slope values for each concentration when plotted versus $Hr^{0.5}$ calculated by linear regression test using GraphPad Prism™ Software

	0.5%	**1%**	**2%**
R2	0.9963	0.9883	0.9972
Slope	0.407 ± 0.008 µg/$Hr^{0.5}$	1.009 ± 0.033 µg/$Hr^{0.5}$	1.043 ± 0.017 µg/$Hr^{0.5}$
Equation	Y = 0.4072*X + 3.319	Y = 1.009*X + 6.641	Y = 1.043*X + 6.867

Release from both the 1% and the 2% groups showed similar behaviour. This is suggested to be due to over-saturation of the drug for concentrations above 1% (w/w). Thus a 1% (w/w) concentration was chosen as a starting point for the *in vivo* safety experiment.

3.7. In vivo studies

The *in vivo* experiment was performed using FVB mice and was undertaken to explore the safety of topically applying Alisertib in the vaginal setting. All protocols and procedures were approved by the Animal Ethics Committee (GU Ref No: MSC/06/16/AEC) prior to commencing animal work.

3.7.1. Animal Procedures

Fifteen mice were randomized equally into three groups: control, placebo and treatment. Placebo rings were manufactured based on the previously explained method, excluding the drug powder (section 2.6.2). The concentration of 1% (w/w) Alisertib rings was chosen as a starting point for the treatment group and the control group did not receive any rings.

Weights of the mice were recorded before the rings were implanted following the previously described method. Daily weighing and visualising were performed to identify any significant changes in behaviour, health or weight. Figure 3.25 depicts the change in weight of the groups over the study period.

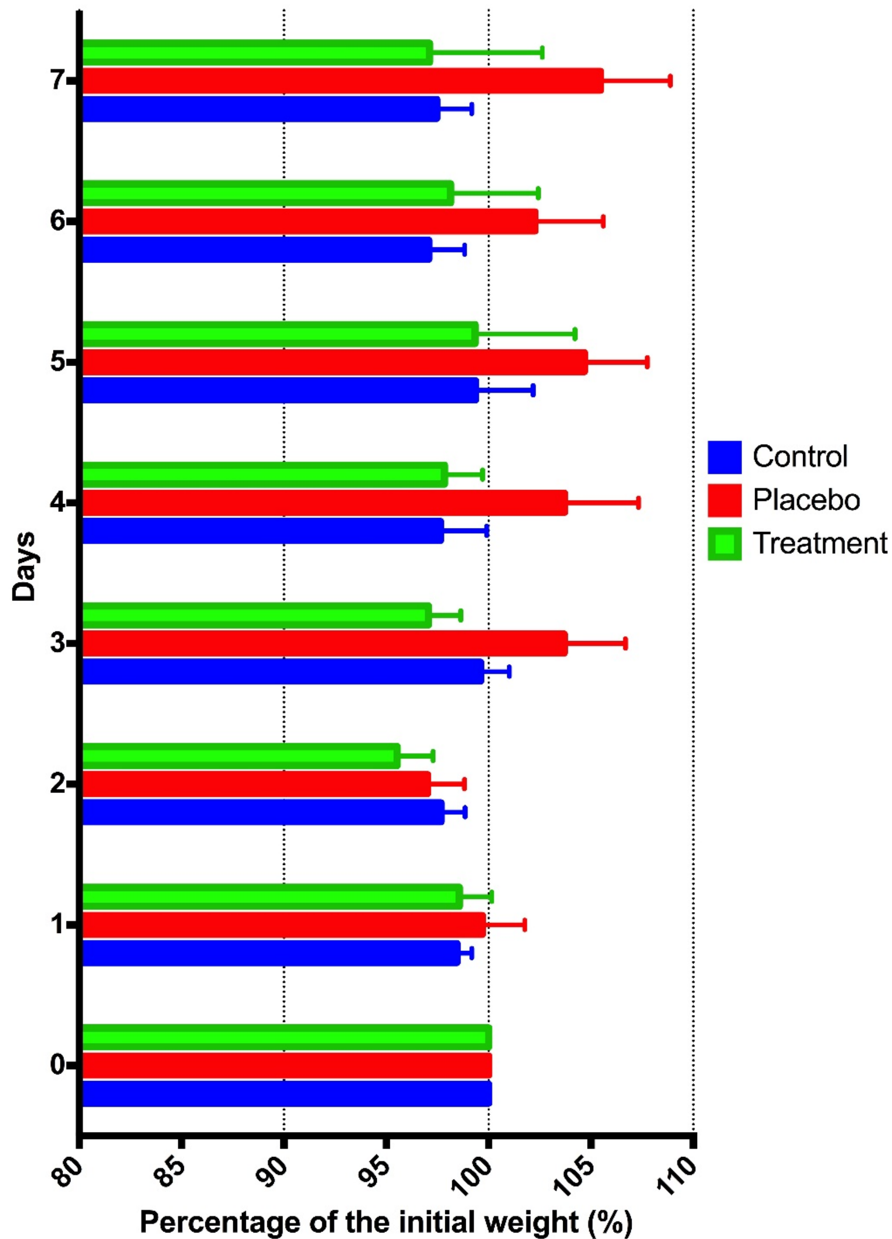


Figure 3.25. Change of weight in the three groups of mice over the study period. 100% represents the initial weight

3.7.2. Statistical Analysis for the Change in Weight

In order to detect any significant difference in the weight change among the placebo or treated groups, multiple t-tests were performed to compare the means of each group daily with the control group.

The multiple t-tests showed no significant difference in the change of weight for the placebo or the treated group compared to the control during the duration of the experiment. The results of the multiple t-tests are summarized in table 3.7.

Table 3.7. Multiple t-tests comparing the weight of the placebo and treatment group individually to the control group over the study period. p value was not significant during the 7 days.

Day	Control (n=5) Mean(SD)	Placebo (n=5) Mean(SD)	Treatment (n=5) Mean(SD)	Placebo/Control		Treatment/Control	
				T	P value	T	P value
1	98.46(1.62)	99.70(4.62)	98.57(3.55)	-0.57	0.586	-0.06	0.950
2	97.67(2.63)	97.02(4.04)	95.53(3.92)	0.30	0.769	1.01	0.340
3	99.60(3.14)	103.69(6.71)	97.06(3.54)	-1.23	0.252	1.20	0.263
4	97.65(4.52)	103.71(8.09)	97.85(4.15)	-1.33	0.225	-0.07	0.947
5	99.37(5.62)	104.68(6.89)	99.34(10.91)	-1.24	0.254	0.004	0.997
6	97.07(3.51)	102.26(7.46)	98.14(9.58)	-1.27	0.245	-0.21	0.839
7	97.47(3.42)	105.47(7.63)	97.12(12.31)	-1.93	0.095	0.05	0.958

3.7.3. Sectioning of the genital organs did not show significant drug-related increase in abscesses or inflammation

Sections of the female genital tract of the three groups of mice (control, placebo and treated) were examined. There was a moderate infiltration of neutrophils as well as other chronic inflammatory cells in all of the tissues. This inflammation was mostly in the sub-epithelial stroma of the mucosa of the vaginas. In some areas, active inflammation was noted in the squamous epithelium of the vaginas.

In some regions, there was a mild increase in inflammatory cells among the groups of mice with insertion of rings (both the placebo and the treated groups). However, there was no significant changes in the degree of inflammation or forming of abscesses (figure 3.26). Therefore, we conclude that the insertion of the rings results in some inflammation but Alisertib does not appear to induce any side effects, inflammation or cell destruction during 7 days of release and exposure.

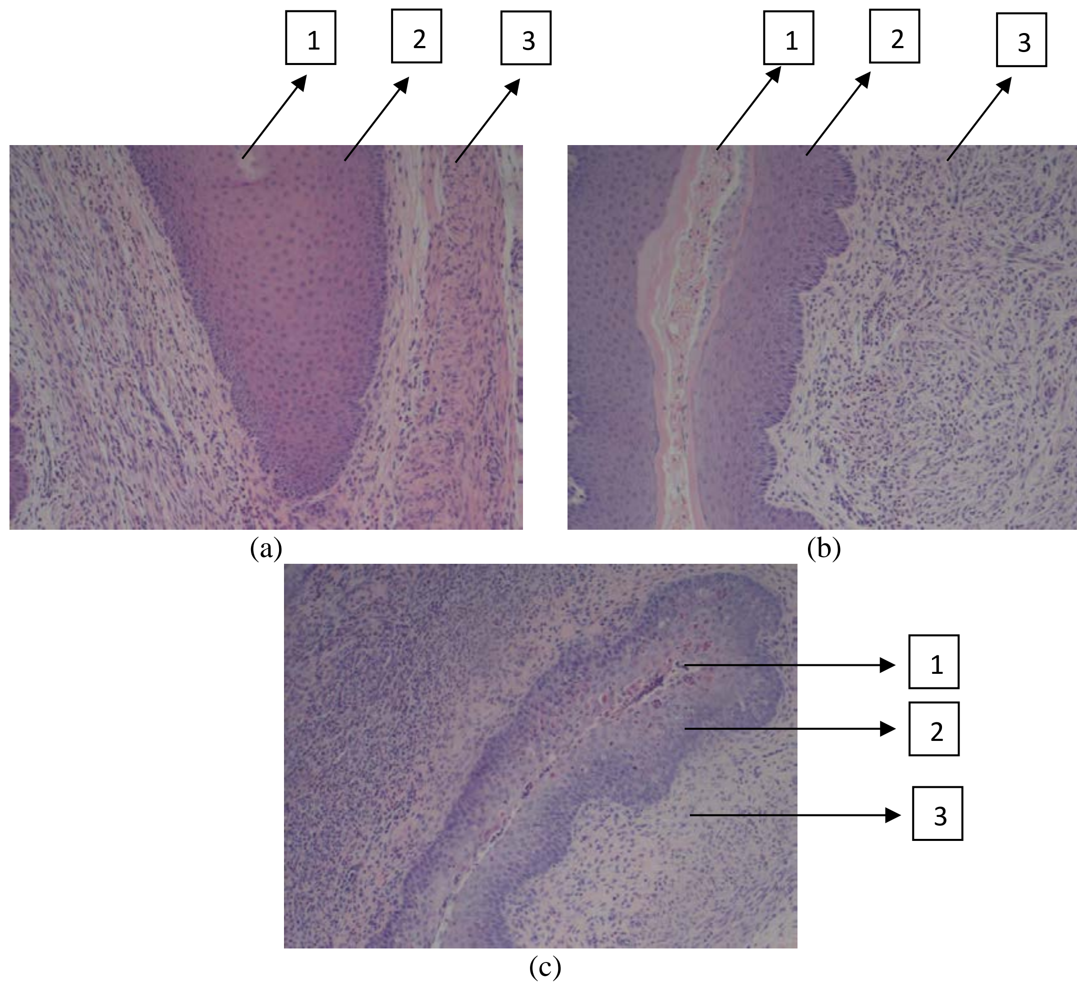


Figure 3.26. Sectioning of the genital organs did not show significant drug-related increase in abscesses or inflammation. (1) is the vaginal lumen, (2) is the epithelium, and (3) is the lamina propria. Mild increase in inflammatory cells among the groups of mice with insertion of rings (both the placebo and the treated groups) was noticed, but no significant changes in the degree of inflammation or forming of abscesses was recorded (a) control Goup (b) placebo Group (c) treated Group.

Chapter 4: Discussion and Conclusions

In this study, we investigated the potential of topical delivery of Alisertib, the Novel Aurora A kinase Inhibitor, for the treatment of cervical cancer. Although, topical delivery to the cervix is feasible, it was necessary to explore and optimise biological, chemical and technical aspects of the chosen delivery platform to obtain the optimal characteristics, before we could address the issue of actual treatment.

In the current research, it is suggested that topical delivery to the cervix for the treatment of cervical cancer is an underexplored area that has the potential to significantly improve therapeutic outcome. Delivering active ingredients directly to their site of action could result in better responses, fewer side effects and use lower doses than that of systematic delivery due to directly exposing the malignant cells to the compound of interest.

A key benefit of using topical delivery is avoiding first-pass organs such as the liver, spleen, and lungs, thus avoiding systemic toxicity. The latter is an important consideration when studying molecules that may have unfavourable systematic safety profiles, as shown above. For cervical cancer, it could be hypothesised that the feasibility of delivering drugs through the vaginal tract makes it an excellent target for topical delivery of potential compounds, especially if systemic administration results in serious side effects. More precisely, the early stages of cervical cancer, where the malignancy is confined to either the cervix or the vaginal tract (stages I to II), where 75% of new cases are diagnosed [(51)], would greatly benefit from this approach.

The compound investigated here, Alisertib (MLN8237), has been shown to have some serious adverse events (mainly neutropenia) associated with the oral administration of the drug following phase I/II trials [(82, 85-90)]. Despite the reported side

effects, it has been used in more than 100 trials for a range of tumours up to phase III. Interestingly, our laboratory has identified that this molecule is selectively lethal when used to treat E7-expressing cells. As more than 99% of cervical cancer cases are caused by HPV, which is dependent on expressing E7 in order to survive [(40), section 3.1.1], it was logical to investigate localised delivery of Alisertib.

Given the results of our previous animal studies (40), we would aim for a complete regression of the disease if treated at early stages. For more advanced cases, this treatment will be applied as neoadjuvant treatment (given before surgery to reduce the size and aggressiveness of the malignancy) before full regression is achieved by combining this therapy with radiotherapy and surgery, and/or as follow-up treatment after surgery to prevent recurrence of the disease.

As a starting point, a potential platform of delivery was identified. We selected silicone intra-vaginal rings for their ability to deliver hydrophobic drugs (like Alisertib) over an extended period of time based on the reliability achieved by those currently commercially available (104, 105).

To date, 5 intravaginal ring products have reached market, all of which were indicated for either contraception or estrogen replacement. Silicone elastomers were used in 4 of these rings. This is mainly due to their biocompatibility, flexibility and light weight (104, 136). On the other hand, the hydrophobic properties of silicone elastomers limited their application to hydrophobic compounds like steroids. Therefore other materials like Polyethylene vinyl acetate (pEVA) and polyurethanes, have emerged to increase the compatibility with a broader range of more hydrophilic compounds (116, 137-143). As Alisertib is a highly hydrophobic compound (logP=5.74, as

predicted using MarvinSketch Software, ChemAxon), it was logical to first begin with testing the ability to incorporate it in silicone, the historical polymer of choice.

Having satisfied ourselves of the importance of addressing a topical and effective delivery system for cervical cancer, in addition to nominating a potential drug and suitable delivery platform, it was essential to investigate how to put the drug and the delivery system together and address the many complications that may arise in such drug development process.

The first aim was to ensure Alisertib was able to retain activity in the vaginal tract and during ring incorporation. Thermal gravimetric analysis (TGA) was described in literature as a way of testing thermostability [(144)]. However, a biological assay was used here (cell viability) in order to ensure that the drug retained its biological activity against cervical cancer cell lines, not only the physiochemical properties.

Interestingly, the biological efficacy of Alisertib showed high tolerance to all conditions related to exposure to the simulated vaginal fluids (acidity) and the process of embedding the drug into silicone rings (elevated temperature) [(sections 3.1.2 and 3.1.3, respectively)]. Simulated vaginal fluid was used as it has been widely studied in literature to test vaginal rings. The accurate compositions of this medium were firstly described by *Owen* and *Katz* [(133)], and all aspects that may affect interactions between the drug and media, including osmolarity and pH, were controlled in this fluid.

Combining these findings with the previously discussed efficacy, the suitability of Alisertib as a candidate for incorporation into silicone-based intra-vaginal rings for topical delivery in the vaginal tract was further supported.

Another important consideration when studying intra-vaginal rings is measuring and optimising their release profile. This study is essential to predict the behaviour of the rings in the vaginal setting. In other words, the concentration of the drug to be incorporated, the design of the rings, plus the length and cycles of the treatment, are all variable factors that are optimised depending on the release profile and how it relates to the both the effective dose and the maximum tolerated dose.

It might be too early to target a specific release profile, as no previous studies have treated cervical cancer topically with Alisertib. However, it was logical to initially target concentrations slightly above the IC_{50} identified *in vitro* [(section 3.1.1)]. This will be optimised in a later experiment in a larger animal model, like sheep.

Many considerations contributed to designing and testing the current rings. Firstly, the medical grade silicone elastomer used in fabrication is limited to external use or short term implant applications ≤ 29 days. Therefore, the release profile was tested initially over 21 days. Secondly, we were interested in testing the safety of a high loading dose followed by continuous release for 3 weeks as we believe this will lead to a more rapid initiation of the drug's effect. Matrix type vaginal rings showed the phenomenon "burst effect" (high loading dose followed by continuous release) in many previous publications, and were therefore chosen.

Historically, studying the release profile of vaginal rings was undertaken in the majority of the developed rings [(104, 116, 137-143, 145-150)]. Either SVF or mixtures of isopropanol/water was used as the release medium for the rings. The latter medium has been widely used in the release testing, as it is more effective than aqueous medium as SVF in dissolving insoluble compounds like Alisertib [(151-153)]. HPLC quantification method was developed for each compound to measure the daily release.

Developing a reliable method of quantification was therefore essential. A selective and sensitive HPLC quantification method was developed to quantify Alisertib in its solutions. The developed method was found to be reliable in quantifying Alisertib in Acetonitrile (the standard solutions) and 50% isopropanol/water solutions (the release medium of the rings). However, there was a flaw in quantifying the compound in simulated vaginal fluid (SVF) solutions.

No such issue was reported in the literature, so it may be argued that the drug degrades in the acidic media of the SVF. Nevertheless, the results from the biological efficacy after exposure to vaginal fluids [section 3.1.2], in addition to the inconsistent pattern of measurement over time (figure 3.12), suggest that the problem was due to the extraction method of the HPLC. Undetectable forms of Alisertib when present in SVF solutions using HPLC were therefore suggested and explored.

Factors that might change the physiochemical characteristics of the compound, such as the acidic and hydrophilic nature of the SVF, are believed to play a major role in eluting a portion of the compound with the solvent front in the HPLC chromatogram, and as a result prevent it from being detected. The effect of hydrophilicity was further tested by extracting the drug from SVF into the organic HPLC mobile phase (Acetonitrile) before running the measurement. This procedure theoretically allows higher amounts of the compound to present in the organic phase and therefore be detected in the HPLC chromatogram at the same original retention time. The improvement achieved following this procedure [section 3.3.3], supports the suggestion that hydrophilicity affected the retention time of the compound.

In regards to acidity, raising the pH of the samples using 0.1 M NaOH has also significantly affected the extraction efficiency of the developed HPLC method [section

[3.3.4)]. Altering acidity mainly affects the distribution of the compound's micro-species [(144)]. Therefore, the early elution may be explained by the distribution of the micro-species of Alisertib, depending on the pH. Studying micro-species of drugs was also recorded in the literature as a way of predicting solubility in the release medium [(144)].

The major possible micro-species of Alisertib were generated using MarvinSketch Software (ChemAxon) version 16.10.3.0 and illustrated in figure 4.1. Over different values of pH, Alisertib can behave either as a weak acid or a weak base, and therefore the distribution of the micro-species varies over different pH values (figure 4.2. Generated by MarvinSketch Software (ChemAxon) version 16.10.3.0).

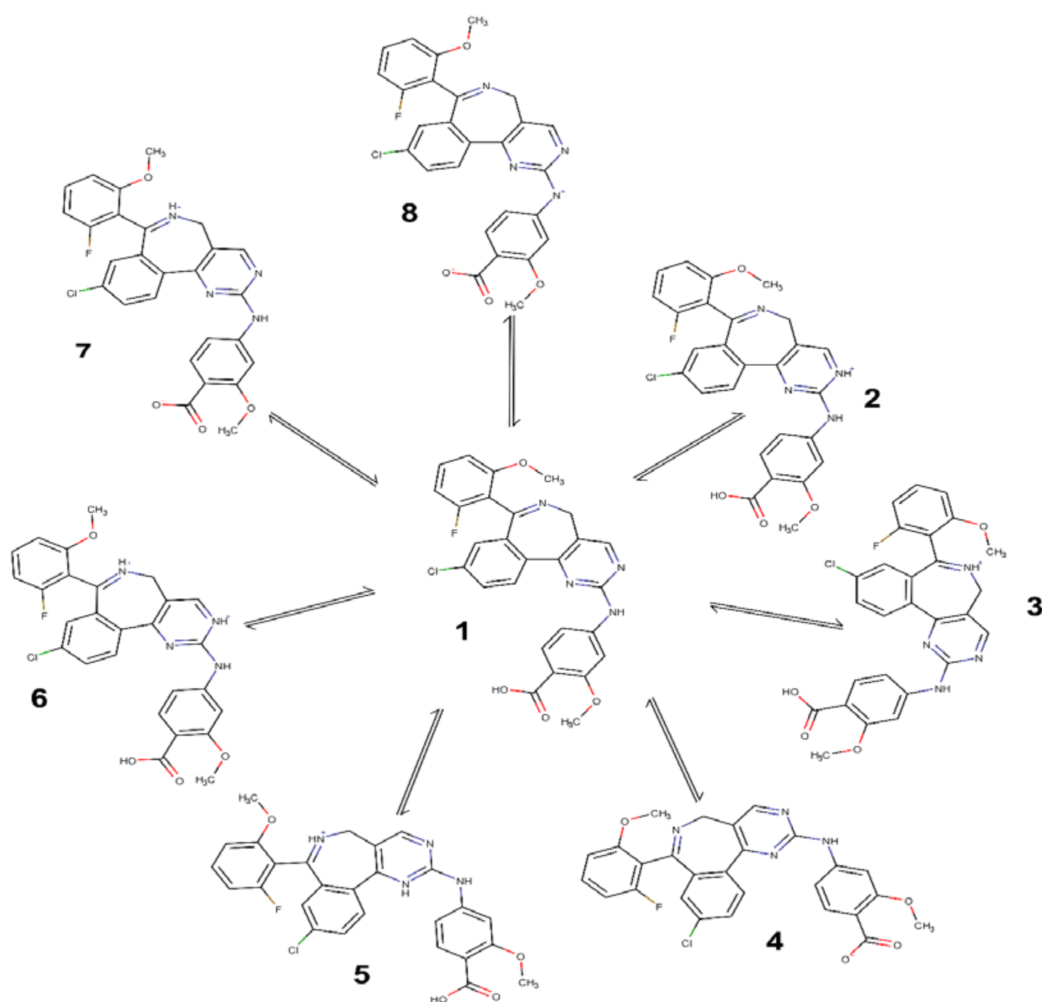


Figure 4.1. The major possible micro-species of Alisertib. Generated using MarvinSketch Software (ChemAxon) version 16.10.3.0.

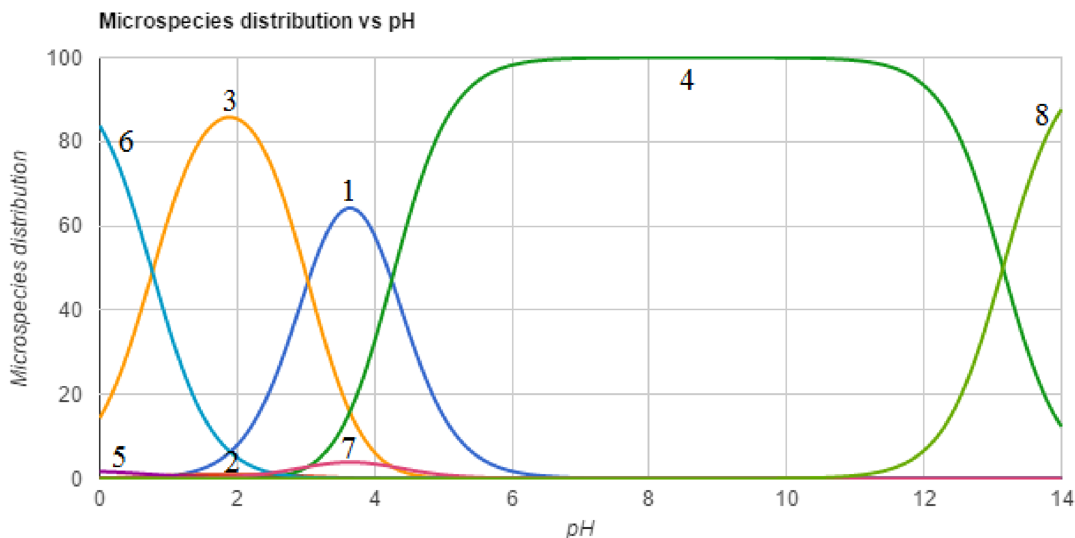


Figure 4.2. The distribution of the possible micro-species of Alisertib over different values of pH (0-14). Generated using MarvinSketch Software (ChemAxon) version 16.10.3.0. Numbers on the graph represent micro-species in figure 4.1.

While C18 column (non-polar chains of Octadecyl carbon) was used as a stationary phase, the increased polarity of some of the above micro-species in a polar media like SVF, may have resulted in a short retention time of the compound, which would result in an early elution during the solvent front's peak.

To test this theory, LC-MS method was developed (section 3.3.5), and elution of the drug ions was detected over the whole run duration. The data from the mass-spectrometry showed a significant undetected portion of the drug eluted during the HPLC run in SVF solutions (figure 3.17), while no such early elution was detected in Acetonitrile and 50% isopropanol/water (figures 3.16.a and 3.16.b, respectively).

To sum up, the developed HPLC method was found to be reliable in quantifying Alisertib in many solvents, like acetonitrile and 50% isopropanol/water, however the previously discussed data almost certainly confirmed that measurement of Alisertib concentration using the developed HPLC method is not possible in the SVF solutions at this stage, and another extraction/quantifying method is to be developed for SVF if

needed. Release profile of the rings was assessed in a mixture of 50% isopropanol/water only.

Measuring the release in these mixtures was found to be reliable, as no early elution was detected in mass spectrometry [section 3.3.5], nor did the measured amounts show inconsistency over time [section 3.5]. However, previous studies of the release profile of intravaginal rings highlighted the importance of maintaining sink conditions (drug concentration in the release medium should not exceed 10% of saturation) for better predicting the behaviour of the rings in the actual vaginal settings [104, 105, 116, 135]. Therefore, 3 ml of the release medium was tested to ensure that the release amount would not exceed 10% of saturation (24.63 µM) at any time.

The release profile of three rings concentration (w/w) (0.5%, 1% and 2% drug/silicone) was assessed over 3 weeks. “Burst effect” was expected and noticed in all ring concentrations, where a relatively high amount of the drug is released over the first one to two days. This phenomenon is well documented and is a result of the release of the dissolved and solid drug’s molecules present in the outermost layer of the rings immediately after being incubated in the release medium [104].

The “Burst effect” may be favourable when high loading dose of the drug is required. It may, on the other hand, generate unwanted side effects due to the high initial concentrations. This can simply be avoided by encapsulating the rings with a drug-free layer of silicone (reservoir-type rings) which controls the release and prevents this effect [116, 138, 154, 155]. However, we aimed at this stage, to test the safety of this concept and modify the platform at a later stage, based on the safety data.

It was also noticed that the release from the 1% and the 2% rings behaved similarly over the study period, which may be explained by the oversaturation of the silicone at

concentrations above 1% (w/w). Over longer periods of release, concentration of the drug dissolved in the silicone will get lower and the 2% drug loaded rings were expected to keep their saturation for longer periods. Therefore, the 2% group might have the ability to retain their release profile for extended durations and would have been considered if a more extended duration of release was required. However, as mentioned earlier, the medical grade silicone elastomer used in fabrication is limited to external use or short term implant applications ≤ 29 days, and any longer release profile would not be desirable as it would potentially be toxic to normal cells. Thus, the development of the 2% concentration was not further investigated.

It was also observed that crystalline drug existed in the rings. Similar cases in previous studies exhibited root time kinetics ($t^{1/2}$), which indicated that the release rate of the Alisertib was controlled by molecular diffusion through the silicone elastomer [104]. Interestingly, the release profile of Alisertib silicone rings obeyed root time ($t^{1/2}$) kinetics, confirmed by a linear cumulative release versus square-root time profile (figure 3.24). When more drug gets released, solved particles will diffuse through the silicone and be released through the surface of the rings into the surrounding medium, allowing undissolved drug particles to dissolve in the depleted sites. As a result, an equilibrium between dissolved drug in silicone, solid drug in silicone, and dissolved drug within the surrounding release medium maintains a linear release of the drug versus square root time as long as more undissolved drug exists within the rings [100, 104, 105]. Studying these trends combined with the safety data, will help deciding the targeted treatment cycles.

Having discussed the release profile of the rings, the final consideration as outlined earlier aimed to address the general safety of administering this delivery device in actual vaginal settings (the effect of the drug on healthy tissues). As a starting point,

the first animal experiment was set to identify any serious inflammation in the vaginal tract resulted from this topical delivery of Alisertib over a short period (one week). One ring concentration (1%) was tested in this experiment. This aimed to provide guidance to study further concentrations based on the safety of this concentration.

Whilst all rings were implemented successfully, a number of issues with the protocol were identified. Firstly, previous studies that implanted vaginal rings in mice used suturing to secure the rings in place (156, 157), while no suturing was used in our experiment, as we hoped to cause minimal procedure-related disturbance to the vaginal tract. This resulted in loose positioning of the rings within the vaginal tract of the mice, which may have allowed them to drop the rings easily. Secondly, it was hard to visualize the rings' positioning over the 7 days, mainly due to the small size of the vaginal openings of the mice during some stages of the menstrual cycle (158).

All rings were able to be visualised and confirmed to be in place either on day 3 or 4 but no rings were found left in the vaginal tracts after sacrificing the animals and dissecting the genital organs on day 7. These factors suggest that the results of this experiment are most reliable up to day 3/4.

Knowing the fact that the highest amount of the drug is released within the first couple of days (the burst effect), it was of high importance to study the safety over a short period of the initial application (one week), to assess the effect of this high loading dose. The drop in weight was recorded in the three groups (no statistical significant difference between the groups' means), which may be explained by the stress experienced by the mice due to the handling and the implanting procedure, which was simulated in the control group as well.

A pathology expert opinion (Prof. Alfred Lam) confirmed no anatomical changes or drug-related elevation in inflammation. These preliminary results encourage taking this platform for further testing and longer treatment cycles. Similarly, Alisertib caused minimal disturbances to the mucosa of the stomach in phase I/II clinical trials (section 1.3.2).

In conclusion, we have shown that Alisertib is a suitable candidate for vaginal delivery using intra-vaginal rings, and the use of this molecule for topical treatment is justified. Safety data is still needed to be collected on a more desirable animal model before identifying the issue of actual treatment with this approach.

A selective HPLC method was developed to quantify Alisertib in the release medium. However, a different extraction/quantification method is to be addressed if quantifying in SVF is required.

In vitro, the manufactured rings achieved relatively reasonable release to their sizes over 21 days and exhibited "burst effect" with root time kinetics over an extended period of time. Moreover, *in vivo*, they did not result in drug-related inflammation or anatomical disturbances over the first week of application.

4.1. Limitations of the study

The present research has not been able to establish the release profile of the manufactured rings in simulated vaginal fluid (SVF) as a release medium. Studying the release profile of intra-vaginal rings in SVF may provide a better understanding on what to expect during the *in vivo* settings.

Secondly, two different batches of purchased Alisertib were found to have slightly different physical characteristics, including colour and particle size. Therefore,

grinding using a mortar and pestle was necessary to minimize the difference in the particle size in one of the batches. This might have resulted in inconsistency in the generated data.

Finally, lack of suturing to secure the rings in the animal experiment did not allow accurate results to be generated, as it was not possible to ensure the positioning of the rings in the vaginal tract for the full duration of the experiment.

4.2. Future Direction

Further research is suggested to develop a reliable method of quantifying Alisertib in hydrophilic acidic solutions as SVF. Identifying a method for completely extracting Alisertib from SVF solutions into organic solvents such as Acetonitrile is a promising possibility which was partially explored in the current study [section 3.3.3].

Actual human-sized vaginal rings will next be manufactured, and tested *in vivo* in sheep models. This will allow studying the final human product in an actual vaginal settings before moving forward to Phase I safety clinical trials. In sheep, *in vivo* release will be measured and correlated to the *in vitro* model. Plasma levels of the drug will be measured to investigate for any systemic absorption, and sectioning the vaginal tract of the sheep at the end point of the experiment will aid in identifying any topical side effects of ring administration.

These rings, in addition to placebo ones, will then be taken to phase I safety clinical trials, and randomised over two groups of healthy women to identify any ring-related and/or drug-related side effects on humans, before the issue of the actual treatment for early cases of cervical cancer is tested in phase II/III studies.

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Appendix A

A.1. Databases Search Strategy

DARE, Cochrane Database of Systematic Reviews and PubMed were searched first to identify any previous systematic reviews on the same topic using the terms [alisertib OR mln8237 OR "mln 8237" OR "mln-8237"] and setting the limiters to review articles only. This search yielded 69 results. Titles and abstracts were screened and 65 articles were excluded as not review articles. Three review articles were identified as not relevant to the topic of interest. The last one discussed the scientific rational that support the clinical development of Alisertib. However, it didn't critically assessed the safety of the drug [(159)].

Next, in order to identify the clinical data available on Alisertib, a preliminary search was done of the Medline database via EBSCO HOST of [alisertib OR mln8237 OR "mln 8237" OR "mln-8237"] in the Title field and limiting the search to human trials only in the last 10 years. This search yielded 45 articles and was followed by searching three more databases: CINAHL database via EBSCO HOST, PubMed and Cochrane Central Register of Controlled Trials with the same terms.

Table A.1. Databases Searched, Terms Used, and Limiters to Retrieve Hits from Databases

Type of Search	**Databases Searched**	**Search Terms**	**Date**	**Search Fields**	**Number of Articles**
Systematic Reviews	DARE	alisertib OR mln8237 OR "mln 8237" OR "mln-8237"	2006 – Present	All Fields	0
	Cochrane Database of Systematic Reviews		2006 – Present	All Fields	0
	PubMed		2006 – Present	Title	69
Clinical Data	Medline		2006 – Present	Title	45
	CINAHL		2006 – Present	Title	8
	PubMed		2006 – Present	Title	156
	Cochrane Central Register of Controlled Trials		2006 – Present	Title, Abstract or Keywords	1
Total for Clinical Date					210

Limiters were set to human clinical trials in the last 10 years (2006 – Present) and language of articles was set to English. Table A.1 shows the hits from each database.

Table A.2. Inclusion and Exclusion Criteria for Selecting Studies

Inclusion Criteria	Exclusion Criteria
• Human Trials (RCTs, Cohort …etc.) • Adult Patients • Diagnosed with any type of cancer and treated with Alisertib (MLN8237) • Side effects would be reported • A complete and clear methodology would be presented • Adverse events and serious adverse events are graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE)	• Alisertib is used in combination with other drugs • Paediatric Patients • Less than 40 patients are included in the study • Animal studies or pre-clinical data

The search strategy is outlined in figure A.1. Briefly, duplicate articles were removed before screening titles and abstracts as per criteria outlined in table A.2. That was followed by retrieving the full text of the articles before the methods were analysed to exclude articles not meeting the selection criteria, which resulted in a total of 7 journal articles that meet all the conditions in the inclusion criteria.

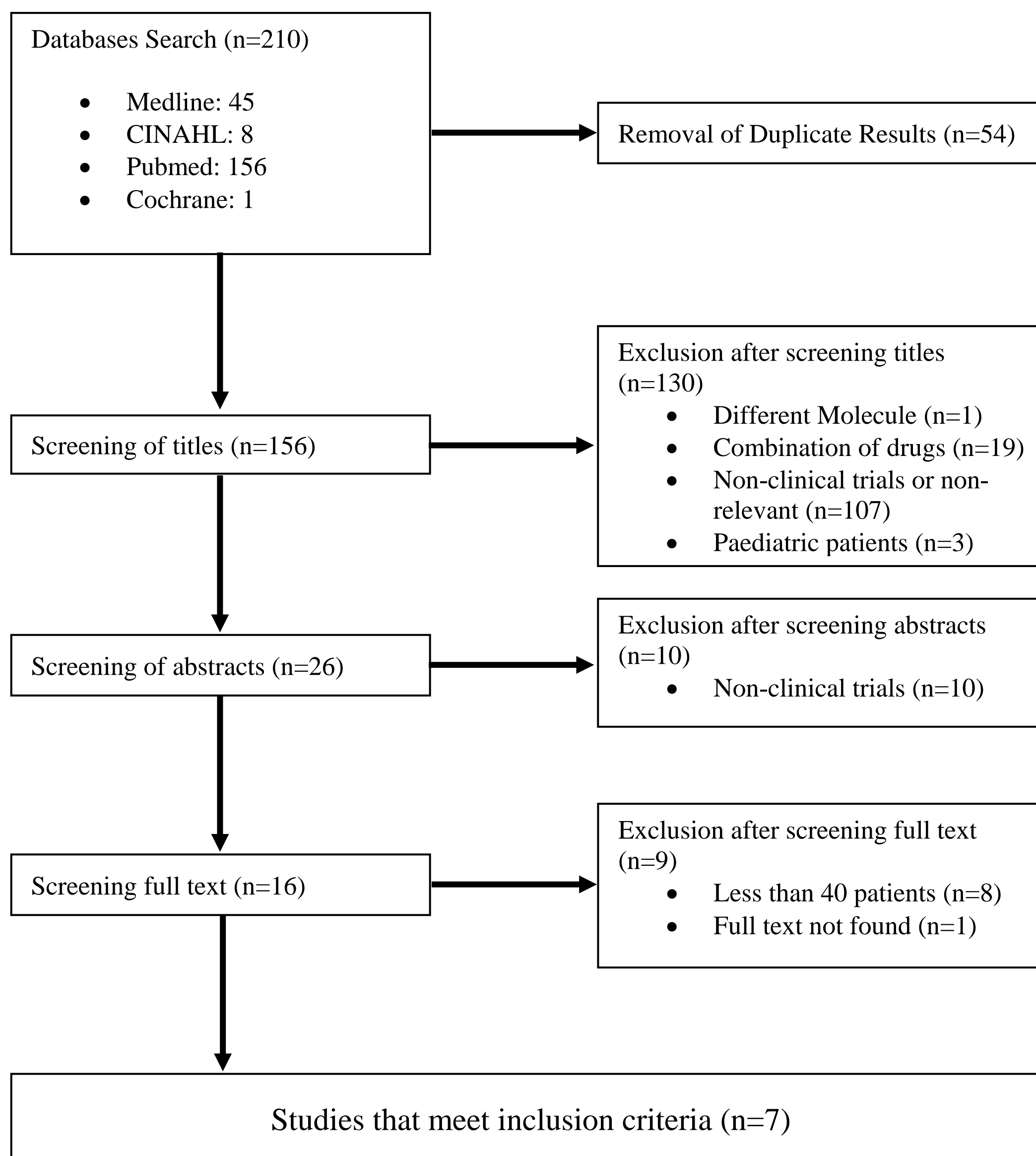

Figure A.1. Search Strategy. After duplicate articles were removed, screening titles and abstracts as per criteria outlined in table 1.2 was performed. That was followed by retrieving the full text of the articles before the methods were analysed.

A.2. Risk of bias assessment for the Selected Articles

Table A.3 shows the titles of the final selected journal articles, the year of publication and the publisher journal.

Table A.3. Articles meeting the inclusion Criteria

ID	Article Title	Journal (Impact Factor)	Year
1	Phase II Study of MLN8237 (Alisertib) in Advanced/Metastatic Sarcoma	Annals of Oncology (9.269)	2016
2	Safety and activity of alisertib, an investigational aurora kinase A inhibitor, in patients with breast cancer, small-cell lung cancer, non-small-cell lung cancer, head and neck squamous-cell carcinoma, and gastro-oesophageal adenocarcinoma: a five-arm phase 2 study	The Lancet Oncology (26.509)	2015
3	Phase I study of MLN8237--investigational Aurora A kinase inhibitor--in relapsed/refractory multiple myeloma, non-Hodgkin lymphoma and chronic lymphocytic leukaemia	Investigational New Drugs (3.281)	2014
4	Phase II study of alisertib, a selective Aurora A kinase inhibitor, in relapsed and refractory aggressive B- and T-cell non-Hodgkin lymphomas	Journal of Clinical Oncology (20.982)	2014
5	An exploratory phase 2 study of investigational Aurora A kinase inhibitor alisertib (MLN8237) in acute myelogenous leukaemia and myelodysplastic syndromes	Leukemia Research Reports (1.19)	2014
6	Phase I study of aurora A kinase inhibitor MLN8237 in advanced solid tumours: safety, pharmacokinetics, pharmacodynamics, and bioavailability of two oral formulations	Clinical Cancer Research (8.738)	2012
7	Phase I pharmacokinetic/pharmacodynamic study of MLN8237, an investigational, oral, selective aurora a kinase inhibitor, in patients with advanced solid tumours	Clinical Cancer Research (8.738)	2012

Quality Assessment Tool for Quantitative Studies was utilized to assess the quality of the included studies [(160)]. Briefly, this tool addresses 8 criteria (selection bias, study design, confounders, blinding, data collection methods, withdrawals and dropouts, intervention integrity, and analysis appropriate to question) to appraise study quality. The final global rating classifies studies into strong (no weak rating in all the 8 areas), moderate (one weak rating), or weak (two or more weak ratings). Discrepancies in quality assessment were resolved by inviting an expert opinion.

The results showed that all the included studies are of a “moderate” global rating (table A.4). Given that all these studies were open-label phase I or II trials, blinding was not possible, and therefore all these studies were downgraded to a “moderate” quality (one weak rating in the blinding criterion). In addition, Melichar *et al* (2051) and Friedberg *et al* (2014) scored “moderate” in the withdrawal criterion as both

studies reported a certain percentage of dropout due to side-effects or other reasons [88, 91].

Table A.4. The quality assessment of the included studies

Studies	Selection Bias	Study Design	Confounders	Blinding	Data collection	Withdrawals and dropouts	Intervention integrity	Analysis appropriate to question	Global rating
Dickson *et al* (2016) [86]	2	2	1	3	1	1	1	1	2
Melichar *et al* (2015) [91]	2	2	1	3	1	2	1	1	2
Kelly *et al* (2014) [87]	2	2	1	3	1	1	1	1	2
Friedberg *et al* (2014) [88]	2	2	1	3	1	2	1	1	2
Goldberg *et al* (2014) [89]	2	2	1	3	1	1	1	1	2
Dees *et al* (2012) [82]	2	2	1	3	1	1	1	1	2
Cervantes *et al* (2012) [90]	2	2	2	3	1	1	1	1	2

1: strong; 2: moderate; 3: weak.