INVESTIGATION OF THE ANTI-PROLIFERATIVE EFFECT OF SYNTHETIC *1-[(Bromomethyl)(phenyl) methyl]-2-(2,4-dinitrophenyl)hydrazine* DERIVATIVE ON CERVICAL CANCER CELLS

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by

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LIST OF ABBREVATIONS

^{0}C	Degree celcius
%	Percentage
g	Gram
g/mL	Gram per milliliter
mg/mL	Milligram per milliliter
mL	Milliliter
µg	Microgram
µg/mL	Microgram per millimeter
µL	Microliter
APS	Ammonium Persulfate
ATCC	American Type Cell Culture
BSA	Bovine Serum Albumin
Cdc	Cell division cycle protein
CDK	Cyclin dependent kinase
CEN	Chicken erythrocyte nuclei
CIN	Cervical Intra Neoplasia
CO_2	Carbon Dioxide
CTN	Calf thymocyte nuclei
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid

FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
HPV	Human Papillomavirus
PBS	Phosphate Buffered Saline
PI	Propidium iodide
RIPA	Radioimmunoprecipitation assay
rpm	Revolution Per Minute
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBEA	Trypan Blue Exclusion Assay
TBS	Tris Buffer Saline
TEMED	tetramethylethylenediamine

PENYIASATAN KESAN ANTI-PROLIFERATIF SINTETIK 1 - [(Bromomethyl) (phenyl)methyl] -2- (2,4- dinitrophenyl) HIDRAZIN DERIVATIF PADA SEL-SEL KANSER PANGKAL RAHIM

ABSTRAK

Kanser pangkal rahim masih kekal sebagai kanser yang kedua tertinggi di kalangan wanita dan punca ketiga utama kematian walaupun pelbagai rawatan sedia ada. Kurang keberkesanan rawatan tersebut telah menyebabkan para saintis mencari rawatan alternatif bagi penyakit ini. Strategi agen anti-proliferatif baru untuk kanser pangkal rahim adalah salah satu inovasi yang penting dalam melawan penyakit yang sengsara ini. Walaupun derivatif hydrazone sintetik telah digunakan secara meluas dalam penyelidikan kanser tetapi mekanisme anti-kanser yang sebenarnya yang ditunjukkan oleh bahan kimia ini masih belum diketahui. Kajian ini cuba menjelaskan kesan-kesan anti-proliferatif1 - [(Bromomethyl) (phenyl) methyl] -2- (2,4- dinitrophenyl) hidrazin ($C_{14}H_{11}BrN_4O_4$) derivatif (kompaun Hydrazone) pada sel-sel kanser pangkal rahim melalui penentuan kepekatan perencatan (IC_{50}), assay anti- proliferatif, assay apoptosis, assay kitaran sel, analisis Western Blot, assay invasi dan analisis cytotoxicity .Hasil kajian menunjukkan dos IC_{50} bagi kompaun Hydrazone adalah 0.03mg/ml pada sel-sel kanser pangkal rahim dan berjaya menghalang proliferasi sel-sel kanser berbanding dengan sel-sel kanser yang tidak dirawat. Di samping itu, aliran sitometri analisis mengesahkan hydrazone derivatif menyebabkan pembantutan sintesis deoxyribonucleic acid (DNA) pada fasa S dan G2 / M tanpa induksi apoptosis. Analisa ‘ western blot’ menunjukkan peningkatan ekspresi protein $p27^{kip1}$ dan PCNA serta merencatkan ekspresi protein Cyclin E2 dan cdc25. Kesan anti-metastasis dikaji melalui analisis

invasi dan ia menunjukkan kompaun hydrazone telah menghalang 50.94 % invasi sel HeLa. Kesimpulannya kompaun hydrazone memiliki kesan anti-proliferatif pada sel HeLa.

INVESTIGATION OF THE ANTI-PROLIFERATIVE EFFECT OF SYNTHETIC *1-[(Bromomethyl)(phenyl) methyl]-2-(2,4-dinitrophenyl)hydrazine* DERIVATIVE ON CERVICAL CANCER CELLS

ABSTRACT

Cervical cancer remains as the second most common type of cancer in women and it is the third leading cause of death despite various treatments that are available. Inefficiency of present treatments had triggered scientists to seek for an alternative treatment for this disease. Development of new anti-proliferative agent for cervical cancer is an important innovation in fighting this miserable disease. Although, synthetic hydrazone derivatives are widely being used in cancer research, the actual anticancer mechanism shown by these chemicals is still not yet understood. Therefore this study aimed to elucidate the anti-proliferative effects of the 1-[(Bromomethyl)(phenyl)methyl] -2-(2,4 -dinitrophenyl) hydrazine ($C_{14}H_{11}BrN_4O_4$) derivative (Hydrazone compound) on cervical cancer cells through determination of inhibitory concentration (IC_{50}), anti-proliferative assay, apoptosis assay, cell cycle assay, western blot analysis, invasion assay and cytotoxicity analysis. The results showed the IC_{50} of the hydrazone compound at 0.03mg/ml on the cervical cancer cells and inhibits the proliferation of cervical cancer cells in a dose and time-dependent manner compared to the untreated control. In addition, the flow cytometry analysis confirmed the hydrazone derivative causes S and G2/M phase cell cycle arrest without induction of apoptosis. Western blot analysis showed that hydrazone compound had increased the $p27^{kip1}$ and PCNA and suppressed Cyclin E2 and cdc25 protein expression. The anti-metastatic effect was studied through invasion assay and it showed the hydrazone compound had inhibited 50.94% of HeLa cells invasion. It

is concluded the hydrazone compound possesses anti-proliferative effect on HeLa cells.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Cancer is a disease that has been discovered since 1500 BC by the Egyptians. The term was introduced by the Greek physician Hippocrates who called it as karkinos, which means crab (Hasan, 2009). Nowadays this disease is been one of the dominating cause of death in developed and non-developed countries (Torre *et al.*, 2015). Cancer able to arise almost in all human organs. The incidence of cancer and death is on a rising from year to year worldwide.

Cervical cancer was one of the most common cancer among women with an estimated incidence of 527, 600 cases and 265, 700 deaths worldwide in the year 2012 (Torre *et al.*,2015). While the National Cancer Registry (NCR) had stated in the year 2007, cervical cancer is been in third rank, with a total of 847 cases diagnosed in Malaysia (Omar and Tamin, 2011). In the year 1943, Pap Smear was developed and since then it has been widely used for the screening of cervical cancer. This test had helped in the reduction of cervical cancer mortality by 70% in the period of 50 years in the US (Almeida & Barry, 2008). Human papillomavirus (HPV) are believed to be strongly related to cervical cancer. This virus used to be one of the main risk factors in this type of cancer and there is more than 200 types of viruses discovered (American Cancer Society, 2015). It is believed that the introduction of the (HPV) vaccine had reduced the incidence of this cancer in most of the developed countries(Lowy & Schiller, 2012).

The diagnostics tools of cervical cancer such as Pap smear and HPV test help to reduce the incidence of this cancer. Furthermore the early detection of this cancer may assist in the identification and selection of treatment to work more efficiently. Nevertheless, there is the necessity for an introduction of better alternative treatment to improvise the prognosis and mortality rate of this disease. Hence, new treatment approaches are required to inhibit the progression of cervical neoplasia before it undergo metastasis.

The approach of finding new treatments for cancer is not only restricted to naturally available resources but synthesized chemicals are also being widely practiced. One of the versatile compounds is hydrazone. The hydrazone derivatives are not only play the role as anti-inflammatory, anti-microbial, anti-HIV but also believed to have anti-cancer properties (Belskaya *et al.,* 2010; Rollas & Kucukguzel, 2007). Previous studies had showed tremendous results of hydrazone derivatives treatments on cancer (Gursoy and Karali 2003; Terzioglu and Gursoy 2003; Zhang *et al*., 2004; Gursoy and Guzeldemirci-Ulusoy, 2007). In this study 1-[(Bromomethyl)(phenyl)methyl]-2-(2,4 -dinitrophenyl) hydrazine ($C_{14}H_{11}BrN_4O_4$) derivative (hydrazone compound) was chosen to act as a treatment to halt the proliferation process. This hydrazone compound had been used to treat tongue cancer cells and shown to act as apoptotic inducer with addition of chemopreventive activity (Zulkepli *et al*.,2011).

Other than that, another prospect of this study is to determine the anti-metastatic effect of hydrazone compound on cervical cancer cells. Once the cancer undergo

metastasis, it is unlikely to find a cure for this disease and often would lead to death. There were few previous studies that introduced drugs and anti-metastatic agents on cervical cancer (Park *et al*., 2010; Liu *et al.*, 2012; Onal *et al.*, 2013; Henderson *et al.*, 2015; Buyukkarabacak *et al.*,2015). Somehow the search for the prevention of metastatic cancer is still not achieved. Thus, there is a requirement for the search of preventive agent on metastasis of cervical cancer. This study had researched the ability of hydrazone compound to suppress the invasion process of HeLa cells.

Somehow the overall progression in cervical cancer showed no dramatic changes even after the presence of few diagnostic, screening and treatments for this cancer (Takekuma *et al.*, 2015). This study offers an understanding on the anti-proliferative effect of the hydrazone compound on cervical cancer cell line.

1.2 Hypothesis and objectives

The hypothesis of this study is that the hydrazone compound induces apoptosis and act as anti-proliferative agent.

The general objective of this study is to learn the chemoprevention effect and anti-proliferative effect of hydrazone compound on cervical cancer cells (HeLa cells). Apart from that, specific objectives of this research include :

1) To determine the inhibitory concentration (IC_{50}) of synthetic hydrazone compound in cervical cancer cells.
2) To identify anti-proliferative effect of synthetic hydrazone compound in cervical cancer cells.
3) To investigate the effect of synthetic hydrazone compound on the apoptosis of cervical cancer cells.
4) To investigate the effect of synthetic hydrazone compound on the cell cycle pathway of cervical cancer cells.
5) To investigate the antimetastatic effect of synthetic hydrazone compound in cervical cancer cells.

The experimental design of this study is shown in a flow chart in APPENDIX A.

CHAPTER 2

LITERATURE REVIEW

2.1 Cervix

2.1.1 Anatomy and Physiology

The female reproductive system consists of internal organs such as uterus, ovaries, vagina and external organs which include the vulva. Cervix is one of the organ of the female reproductive system which is the lower fibromuscular part of the uterus, conical or cylindrical in structure (Tortora, 2009). According to Spencer (2007), the term cervix originates from the Latin word “neck”, since it is long and cylindrical in structure. The cervical tissue connects the uterus with the vagina. Cervix can be divided into two parts, endocervix and ectocervix. The endocervix is the region nearer to the uterus opening and the ectocervix is the part that extends toward the vagina. The endocervix consists of columnar cells which are actually glandular cells that synthesize mucus. While the ectocervix is lined with stratified squamous cells and these cells were consist of multiple layers which are parabasal, basal, intermediate and superficial layer. The cells of endocervix and ectocervix joins to form a squamo-columnar region. The overlapping region is known as the transformation zone (Tortora, 2009). Columnar cells at the transformation zone undergo changes into squamous cells and this region is vulgar to the development of abnormal cells. According to Wylie (2005), columnar epithelium which presents along the cervix secretes mucus in order to synthesize a protective plug against any infection in the internal genitalia. Furthermore, the transformation zone varies

among women throughout their lifetime. Commonly, the transformation zone in teenagers will be on the outer surface of cervix and it is more prone to get infections. While on other hand, the transformation zone will be on the canal of cervix in older women. Human cervix tend to dilate during menstrual flow and dilate widely during childbirth. The cervix can be in various sizes and structures depending on the age of women, hormonal status and parity (Tortora, 2009).

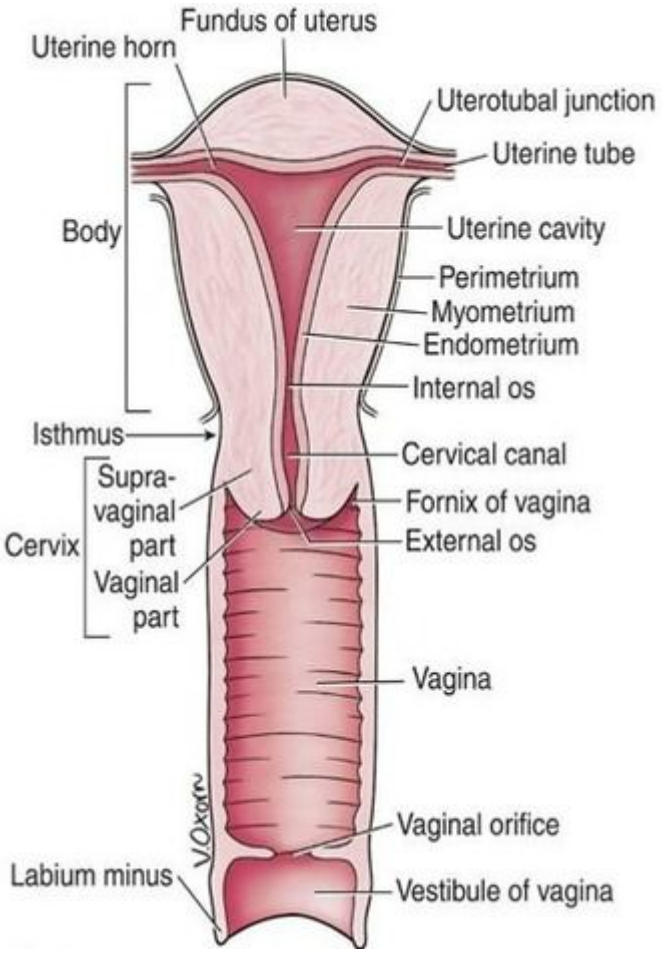

Figure 2.1 : Cross section of uterus and vagina. Adapted from Moore and Agur, 2007.

2.2 Cancer

2.2.1 Epidemology

Cancer is a disease that leads to the uncontrolled mitotic cell division. Cancer can arise from any part of the human body. The normal cells of human usually undergo cell division in a programmed manner and when become old or damaged, the cells will die and replaced with new cells. If cancer develops, the cells will be abnormal and the growth will be in faster rate compared to the normal cells. Furthermore, the cancer cells do not undergo programmed cell death. Cancer cells keep dividing and form tumor. There are two types of tumor, benign and malignant. According to Almeida and Barry (2010), benign tumor does not spread to nearby tissue, has a well defined perimeter and somehow will only be dangerous if the tumor compress surrounding tissues. On the other hand, malignant tumor is able to metastasize to nearby tissues through lymphatic system, blood vessels and even through infected tissues (Dong *et al*, 2009).

Cancer remains as the deadliest disease in the world until today. The incidence of cancer is increasing due to the aging and growth of the population, overweight, smoking, physical inactivity and changing of reproductive patterns (Torre *et al,* 2015). In the year 2014, the number of people who had been diagnosed with cancer reached almost 14.5 million and it is estimated to increase to 19 million by the year 2024 (National Cancer Institute, 2015). Lung, breast and colorectal cancers are in the highest rank as the main cause of cancer death in more and less developed countries (Torre *et al*,2015).

2.2.2 Cancer Carcinogenesis

Multiple steps are involved in the development of cancer from a normal cells. This process of transformation is known as carcinogenesis. The carcinogenesis involves three stages, which include initiation, promotion, and progression (Hennings *et al*., 1993). The experiments which have been done using the animal models showed similar carcinogenesis process in human, somehow due to exposure to various chemical compounds by the human throughout their life do alter the speed of the process and result in different effect (Oliviera *et al*., 2007). Figure 2.2 illustrate the processes of the carcinogenesis, including the three stages that are involved.

Initiation is the first stage of carcinogenesis where the damaged genetic material (DNA or RNA) results in unregulated growth of cells. The initiation stage is reversible and involves both intracellular factors such as inflammation, oxidative stress, inherited genes and hormone, and extracellular factors like exposure to carcinogens, life style and diet (Bertram, 2001). The second stage is the promotion process where a malignant cell is able to reproduce itself and multiply to give rise to large malignant cell numbers. The last stage is the progression which imply growth and expansion of the cells of tumor with potency of metastasis. In the initiation and promotion stage, the cell proliferation and apoptosis take place in different rates but managed to be balanced, but in the progression stage both of the mechanisms are altered and may lead to uncontrolled mitosis (Mehta, 1995).

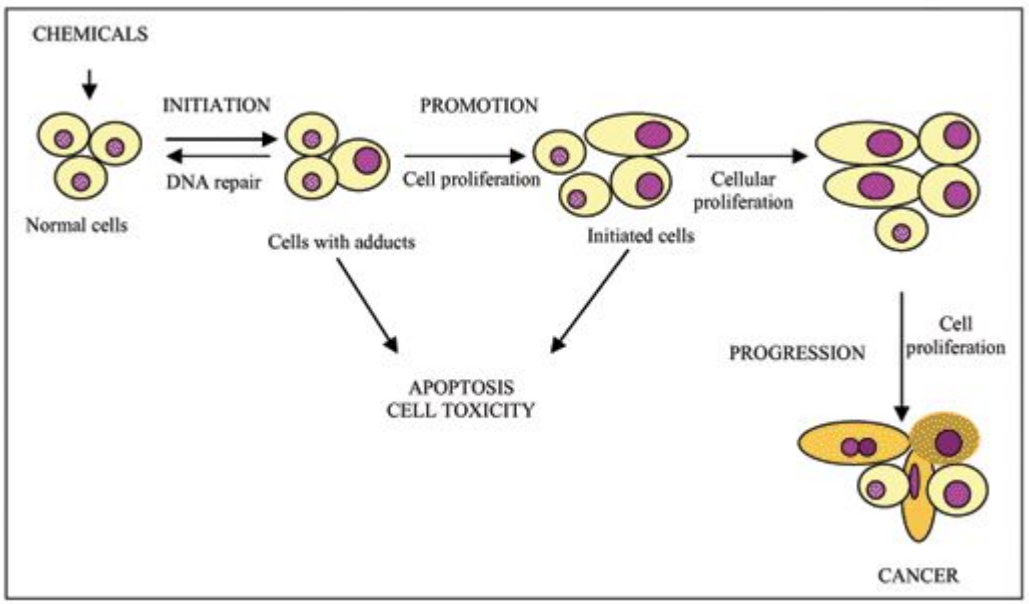

Figure 2.2 : The carcinogenesis stages and the occurences involved in each step. Adapted from Oliviera *et al*., 2007.

2.3 Cervical cancer

Cervical cancer is a slow progressing cancer in the cervix of female reproductive system. This type of cancer does not show an obvious symptoms at the early stage and it begins with a precancerous condition which is known by a few terms such as dysplasia, cervical intraepthelial neoplasia (CIN), and squamous intraepthelial lesion (SIL) (American Cancer Society, 2015). The abnormality growth usually starts in the transformation zone of the cervix. The classification of precancerous and cancerous of the cervix is based on the morphology observed under microscope.

Squamous cell carcinoma and adenocarcinoma are the most periodically diagnosed type of cervical cancer. The squamous cell carcinoma originates in the squamous cells of the cervix, which is the ectocervix region. While the adenocarcinomas begin in the endocervix part. It develops in the gland cells which is responsible to produce mucus. Furthermore, some of the cervical cancers have features of both, adenocarcinomas and squamous cell carcinomas. These type of cancer is known as adenosquamous carcinomas or mixed carcinomas (American Cancer Society, 2015).

Cervical cancer had been in the top three rank in the cancer statistical list. New cases of 11,000 and 4,000 deaths of cervical cancer had been estimated by the American Cancer Society in the year 2008 (Almeida & Barry, 2010). Cervical cancer is in the second rank as the most diagnosed cancer and the third leading

cause of death especially in less developed countries (Torre *et al*, 2015). In less developed countries, the importance and the knowledge on the presence of the screening test and the vaccination of HPV is lacking. In Asia, 144,400 deaths due to cervical cancer has been reported in the 2012 (Torre *et al*, 2015). In Malaysia cervical cancer was the third most common cancer in women with 847 cases been diagnosed in the year 2007 (Omar & Tamin, 2011).

2.3.1 Risk Factors for Cervical Cancer

A few risk factors can lead to an increase in the development of cervical cancer in women. These factors include infections with Human Papillomavirus (HPV), smoking, immunosuppression, overweight and others. In most of the cervical cancer cases HPV infection used to be the main risk factor. HPV are non-enveloped virus with double stranded DNA in circular structure with 8,000 base pairs (Gillison, 2008). There are two higher-order genera, α-papillomaviruses which attack the mucosal epithelia and β-papillomaviruses infect cutaneous epithelia (De Villiers *et al.*, 2004). The papillomaviruses are basically categorized as 'high-risk', 'moderate-risk' and 'low-risk'. The α-papillomaviruses have clearly established as carcinogenic for human (Gillison, 2008).

Smoking is not only one of the major risk factors for cervical cancer but also for all types of cancer. The substances present in the cigarette are harmful and women who smoke are incline to get cervical cancer. Researchers believe that the materials in the cigarette are responsible in destroying the DNA of cervical cells. In addition,

the immune system of a smoker will be less efficient in combating the infections of HPV.

Immune system is plays a very important role in combating the cancer cells. Women who suffer from autoimmune diseases, AIDS and had organ transplant are at higher risk for HPV infections compared to healthy person. Since this group of people have weak immunity toward diseases, they are more easily exposed to cancer. Women who consumes birth control pills for a long period of time increases the risk of cervical cancer. According to the researchers the risk of cervical cancer increases in women who use oral contraceptives. Somehow the risk decreases once they stopped consuming it. Other than that, women who are exposed to diethylstilbestrol (DES) are likely to develop clear-cell adenocarcinoma. Daughters who had their mothers took DES during pregnancy are also at risk of getting precancerous and squamous cell cancers of the cervix. Furthermore, women who have family history of cervical cancer may have chances to develop the cancer (Colditz & Stein, 2004; Almeida & Barry, 2010; American Cancer Society, 2015).

2.3.2 Symptoms and Staging

Women who developed the precancerous or early cervical cancer usually do not have any specific symptoms. The symptoms only present once the cancer turned out to be invasive and starts to spread to nearby tissues. Most common symptoms include unusual discharge of the vagina, pain while having intercourse, pelvic pain and abnormal bleeding of vagina (Almeida & Barry, 2010). Since the symptoms are normally correlated with female reproductive tract diseases, further examinations

and physician consultancy are necessary.

In most cases of cervical dysplasia there are possibility of cure without treatment, however early detection promised better treatment to overcome this type of cancer. If the abnormal cells are left untreated, there is a high possibility that it become malignant (Braun & Anderson,2007). The staging of cervical cancer is based on the size of tumor and the spreading of the cancer cells to other parts of body. The International Federation of Gynecology and Obstetrics (FIGO) has clinically defined cervical cancer (Table 2.3).

Primary tumor (T)		
TNM	**FIGO**	**Surgical-Pathologic Findings**
Categories	Stages	
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Tis		Carcinoma in situ (preinvasive carcinoma)
T1	I	Cervical carcinoma confined to the cervix (disregard extension to the corpus)
T1a	IA	Invasive carcinoma diagnosed only by microscopy; stromal invasion with a maximum depth of 5.0 mm measured from the base of the epithelium and a horizontal spread of 7.0 mm or less; vascular space involvement, venous or lymphatic, does not affect classification
T1a1	IA1	Measured stromal invasion ≤ 3.0 mm in depth and ≤ 7.0 mm in horizontal spread
T1a2	IA2	Measured stromal invasion > 3.0 mm and ≤ 5.0 mm with a horizontal spread ≤ 7.0 mm
T1b	IB	Clinically visible lesion confined to the cervix or microscopic lesion greater than T1a/IA2
T1b1	IB1	Clinically visible lesion ≤ 4.0 cm in greatest dimension
T1b2	IB2	Clinically visible lesion > 4.0 cm in greatest dimension
T2	II	Cervical carcinoma invades beyond uterus but not to pelvic wall or to lower third of vagina
T2a	IIA	Tumor without parametrial invasion
T2a1	IIA1	Clinically visible lesion ≤ 4.0 cm in greatest dimension
T2a2	IIA2	Clinically visible lesion > 4.0 cm in greatest dimension
T2b	IIB	Tumor with parametrial invasion
T3	III	Tumor extends to pelvic wall and/or involves lower third of vagina and/or causes hydronephrosis or nonfunctional kidney
T3a	IIIA	Tumor involves lower third of vagina, no extension to pelvic wall
T3b	IIIB	Tumor extends to pelvic wall and/or causes hydronephrosis or nonfunctional kidney
T4	IV	Tumor invades mucosa of bladder or rectum and/or extends beyond true pelvis (bullous edema is not sufficient to classify a tumor as T4)
T4a	IVA	Tumor invades mucosa of bladder or rectum (bullous edema is not sufficient to classify a tumor as T4)
T4b	IVB	Tumor extends beyond true pelvis
Regional lymph nodes (N)		
NX	Regional lymph nodes cannot be assessed	
N0	No regional lymph node metastasis	
N1	Regional lymph node metastasis	
Distant metastasis (M)		
M0	No distant metastasis	
M1	Distant metastasis (including peritoneal spread; involvement of supraclavicular, mediastinal, or para-aortic lymph nodes; and lung, liver, or bone)	

Table 2.3 : The American Joint Committee on Cancer (AJCC) TNM classification and the International Federation of Gynecology and Obstetrics (FIGO) staging system for cervical cancer.

2.3.3 Treatments for Cervical Cancer

The progression of cervical cancer is very slow, it usually takes few years to develop cancer from a precancerous condition. Somehow the development of cancer can even occur in less than one year. The precancerous condition is able to disappear without any treatment in most women. Treatment of precancerous cells of the cervix can prevent the occurrence of cervical cancer. Treatments that are available for cervical cancer include surgery, chemotherapy, radiotherapy and targeted-therapy.

The first choice of treatment for cervical cancer is the surgery. A few types of surgeries are recommendable for this cancer which include cryosurgery, laser surgery, conisation, hysterectomy, radical hysterectomy, trachelectomy, pelvic exenteration and pelvic lymph node dissection (Jeevan & Olah, 2015). Cryosurgery is a method of placing a metal probe which is cooled using liquid nitrogen directly on the cervix to kill the abnormal cells. It is normally performed to treat carcinoma at stage 0, which is not invasive (Basta *et al,* 2015). Laser surgery is performed using a laser beam which is focused through the vagina to vaporize abnormal cells or to discard a small part of tissue for study purpose. Furthermore, the laser surgery is also been used to treat carcinoma of stage 0. The next one will be the conization, where a piece of cone structured tissue is isolated from cervix using a laser knife or with a thin wire that heated with electricity (the loop electrosurgical, LEEP procedure). The cone tissue biopsy might be used for diagnostic purpose before proceeding with additional treatments. This treatment may also be performed in patient with an early stage of cervical cancer (American Cancer Society, 2015).

Hysterectomy is a surgical removal of the uterus and the cervix. If the removal is done through the surgical incision of the abdomen, then the surgery is known as abdominal hysterectomy, if removed through vaginal it is known as vaginal hysterectomy and if it is through laparoscopy, it is known as laparoscopic hysterectomy. Other than that, there is also radical hysterectomy which removes the uterus including the nearby tissues including upper part of the vagina. The procedure is preformed through abdominal incision and usually pelvic lymph nodes will be removed. This type of surgery is normally conducted for the treatment of stage IA2, IB and IIA of cervical cancer. Furthermore, there is another type of hysterectomy, radical trachelectomy, which treat stage IA2 and IB cervical cancer and grant women the ability to get pregnant even after treatment. The procedure involves isolation of the cervix and vagina without removing the uterus (Musaev *et al*. 2015).

Pelvic exenteration and pelvic lymph node dissection are surgery which is conducted for cancer which had spread. In pelvic exenteration procedure, not only the uterus region will be removed, but vagina, bladder, rectum even a part of the colon might be removed, based on the spreading of the cancer. During the pelvic lymph node dissection, surgeon may isolate some lymph nodes to verify the stage of the cancer (Pomel *et al*, 2003).

The following type of treatment will be the radiation therapy. High energy particles or x-rays is used to kill the cancer cells (Li *et al*, 2015). The therapy can be done externally or internally. External beam radiation therapy (EBRT) involves x-rays beam directed to the cancer from outside of the patient body. The radiation dose is stronger and the treatment is painless. Unfortunately, the treatment may give side effects to the patient. Normally the EBRT procedure will be followed by chemotherapy, therefore the therapy is simultaneously called as chemoradiation. The other type of radiation therapy which is internally practiced is brachytherapy where the radiation source is placed in or adjacent to the cancer. In cervical cancer, intracavitary brachytherapy procedure is performed by placing the source of radiation through a device which is in the vagina or even in the cervix (American Cancer Society, 2015).

The next type of treatment for cervical cancer is chemotherapy, where anti-cancer drugs are injected through the vein or consumed by mouth. These drugs will be introduced to the bloodstream and delivered to all body parts, which then acts on the cancer cells in the entire patient's body. Usually chemotherapy is given in cycles manner which gives a certain period of time for recovery of the patient. Chemotherapy is used in treating cancer which had spread to other parts of the body. There are a few drugs which have been frequently used to treat advanced cancer of cervix including cisplatin, topotecan, carboplatin, paclitaxel and gemcitabine (American Cancer Society, 2015).

Nowadays, due to better understanding on the characteristics of cancer cells and researchers have successfully developed few new drugs which act as targeted cancer treatments. Angiogenesis is the synthesis of new blood vessels and cancer do undergoes metastasis through the help of this process. Angiogenesis inhibitor can be used as a target therapy to immobilize the growth of new blood vessels. In the case of cervical cancer, bevacizumab which is an angiogenesis inhibitor, target the vascular endothelial growth factor (VEGF) that assist in new blood vessels formation (American Cancer Society, 2015). Furthermore, according to American Cancer Society (2016) the addition of targeted therapy in women who are taking standard chemotheraphy treatment had helped to improve the overall survival rate.

2.3.4 Diagnostic tools and tests

Nowadays there are a few screening tests available for an early detection of cervical cancer. The presence of HPV can be analyzed with Pap smear, biopsy, DNA hybridization and Polymerase Chain Reaction (PCR) amplification (Borutto & Comparetto, 2012). Pap smear turns out to be one of the effective test for the detection of cervical cancer during early stage and this tests is available in most hospitals. Currently there are two type of HPV test present, the HPV DNA test which is able to identify 13 types of HPV which are in the category of high-risk and the HPV PCR test which is also used in few hospitals and laboratories (Almeida & Barry, 2010).

2.3.5 Prevention of Cervical Cancer

2.3.5(a) Vaccination

Cervical cancer is a disease which can be completely cured if the abnormal cells were detected at early stage. The presence of diagnostic tools and tests ease the treatment of this cancer. Complementary to this is the discovery of HPV vaccines that give a great promise to decrease the incidence of cervical cancer. Generally preventive vaccine work by neutralizing antibodies toward HPV infections. Two types of vaccines have been introduced for the preventive purpose, 'Gardasil' and 'Cervarix'. Gardasil is a vaccine which targets four types of HPV, which are HPV-16,-18,-6, and -11. HPV types 16 and 18 are observed approximately in 70-75% of cervical cancer, while HPV types 6 and 11 lead to nearly 90% of genital warts. The length of protection of this vaccine is up to 5 years. Cervarix consists of HPV types 16 and 18, which also gives protection for 5 years (Roden *et al,* 2008).

2.4 Cervical Cancer Cell Lines

There are different types of cervical cancer cell lines can be used in research, which include HeLa, SiHa, CaSki, C33A and DoTc2. Each of the cell lines has different origin, characteristics and capability as reported by the ATCC^{R} (American Type Culture Collection) CCL-2^{TM} product sheet. Some are very well established and have been used for years in the research field. C33A cell line exhibited a hypodiploid karyotype intially and epithelial morphology. The cells are negative for HPV DNA and RNA. CaSki cells are derived from metastatic site in the small bowel mesentery and contain HPV type 16 and sequence related to HPV-18. SiHa cells are adherent epithelial cells obtained after surgery from a Japanese patient and is a

suitable host for transfection. HeLa cells are adherent adenocarcinoma cells that are suitable transfection host. HeLa cell is one of the very well established cells in the research field and has successfully been used in few experiments such as polio vaccine development, viruses growth studies, study of cancer causes and cure for leukemia (Almeida & Barry, 2011).

The HeLa cells were isolated from a cervical cancer patient named Henrietta Lacks in the year 1951 (Spencer, 2009). The cells were named after her first two letters of her first and last name. HeLa cell is the first human cell which successfully grow in the in vitro condition. The malignant cells were obtained from her cervix through a quarter sized tumor, where by that time the cancer already spread to most of her body parts (Almeida & Barry, 2011). The cells are adherent cells of the cervix epithelial adenocarcinoma. A report by the ATCC^{R} (American Type Culture Collection) CCL-2^{TM} product sheet, the cells contain HPV-18 strain. The cells are being used widely in research and the cells keep multiply aggressively until today.

2.5 Hydrazone

2.5.1 Background of Hydrazone compound

A lot of different treatments have been used in the new search of cure for cancer. There are naturally present sources and synthesized chemicals widely used as the treatments. One of the synthesized chemicals which has potency to act as an anti-cancer treatment is the hydrazone. There are varieties of chemically synthesized hydrazone derivatives, depending on the functional group attached. Basically

hydrazones have two bonded nitrogen atoms with different nature with a C-N double bond that is conjugated with a single electron pair at the nitrogen terminal (Belskaya *et al*, 2010). Moreover, according to Belskaya *et al* (2010) hydrazones are easily prepared, have tendency toward crystallinity and increased hydrolytic stability compared to imines and these are the main positive traits which encourage to learn more about this compound.

Hydrazones exhibit physiological activities when used as a treatment in several diseases (Monfared et al, 2007). Furthermore, Rollas and Kucukguzel (2007) stated that hydrazone derivative is one of crucial class of chemicals which poses anti-cancer properties. Previous studies had shown the anti-cancer effects of different hydrazone derivatives, such as 3-[[(6-chloro-3-phenyl-4(3H)-quinazolinone-2-yl) mercaptoacetyl] hydrazono]-5-fluoro-1H-2-indolinone which demonstrated cytotoxicity effects on renal cancer cell line (Gursoy and Karali 2003), (2,6-dimethyl-N-(2-hydroxyphenyl-methylidene)imidazo[2,1-b][1,3,4]thiadiazole-5-carbohydrazide showed effects on cancer cells of the ovary (Terzioglu and Gursoy, 2003) and 5-chloro-3-phenylindole-2-carboxylicacid(4-nitrobenzylidene) hydrazide which proven to inhibit the growth of breast cancer cells (Zhang et al., 2004).

One of the hydrazone derivatives is the 1-[(Bromomethyl)(phenyl)methyl]-2-(2,4-dinitrophenyl) hydrazine ($C_{14}H_{11}BrN_4O_4$), which consists of two crystallo-graphically independent molecules which are in the asymmetric unit (Salhin et al,2009). This hydrazone compound have been previously used to treat tongue cancer cell line and proven to act as a potential chemopreventive agent (Zulkepli et

al.,2011). This hydrazone compound had been chosen in this study to investigate its potential and anti-metastatic properties against cervical cancer in *vitro.*

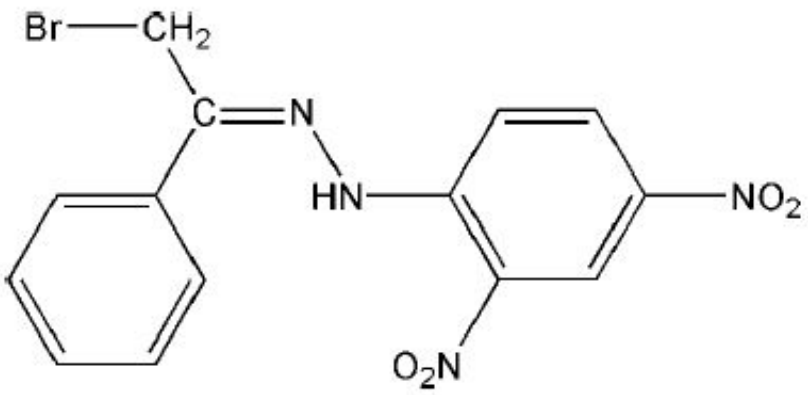

Figure 2.4 : The molecular structure of the hydrazone compound. Adapted from Salhin *et. al*, 2009.

2.6 Metastasis

2.6.1 Overview of Metastasis

The term metastasis refers to the dissemination of cancer cells from the primary tumor to other parts of the body. Metastasis remains as one of the main contributors towards mortality and morbidity of cancer cases (Pienta *et al*, 2013). According to Gupta and Massague (2006), for centuries cancer biologists believed that metastasis process resulted from the interactions of tumor cells with the permissive target tissues. The cancer cells which undergo metastasis will look similar as the original or the primary cancer when observed under microscope (Dong *et al*, 2009). Furthermore, the molecular characteristics between the metastatic cancer cells and the original cancer cells are usually similar, such as the presence of particular chromosomal changes and expression of specific proteins (National Cancer Institute, 2013). Cancer which is formed through these processes are known as metastatic cancer and the tumor is called metastatic tumor.

Metastasis process starts in the last stage of tumorigenesis (Lim *et al*, 2004). According to National Cancer Institute (2013) basically there are series of processes involved in cancer cell metastasis which includes local invasion, intravasation, circulation, arrest and extravasation, proliferation and angiogenesis. Cell motility plays a key role in the progression of cancer to become invasive and undergo metastasis (Lang *et al*, 2005). The cancer cells start to into on surrounding tissues and travel to different region of the body with the help of the bloodstream and lymphatic system. When the cells are arrested or stop moving when reached the blood capillaries, they intrude on the capillaries wall and travel to nearby tissues.

Cancer cells starts to proliferate at distant location to form micrometastases. Later there will be angiogenesis process which is the synthesis of new blood vessels as the source of obtaining necessary needs such as oxygen and nutrients by the cancer cells to continue growing. Treatments are available for all patients with metastatic cancers, unfortunately most of the metastatic cancers cannot be cured with the present treatments. The main purpose of metastatic cancer treatment is to stop the spreading of the cancer from primary tumor to other parts of body. Furthermore, metastasis is one of the major reasons for the death of cancer patients (National Cancer Institute, 2013).

Better understanding on the molecular sequence in various stages of metastasis will help to identify the target of therapies (Dong *et al*., 2009). Malignant cancers always have higher affinity to undergo metastasis process. This includes cervical cancer which is one of the threat for women worldwide and previous studies had discussed the possibilities of treatments for the cervical metastasis (Lim *et a*l., 2004; Yuan *et al*., 2008; Rah *et al*., 2012; Song *et al*., 2013; Li *et al*., 2014).