Anticancer Effects of New Heterocyclic Compounds on Cervical Cancer Cells in Vitro

تأثير مركبات حلقية غير متجانسة جديدة على سرطان عنق الرحم عملياً

By

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Ph.D. Molecular Cell Biology

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September/2018
Anticancer Effects of New Heterocyclic Compounds on Cervical Cancer Cells in Vitro

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نتيجة الحكم

بناءً على موافقة عمادة البحث العلمي والدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحثة/ هالة اسماعيل محمد القاضي لنيل درجة الماجستير في كلية العلوم/ برنامج العلوم الحياتية/ علم الحيوان موضوعا:

تأثير مركبات حلقية غير متجانسة جديدة على سرطان عنق الرحم معملاً

Anticancer effects of new heterocyclic compounds on cervical cancer cells in vitro

وبعد المناقشة التي تمت اليوم السبت 9 صفر 1440هـ الموافق 20/10/2018م الساعة الثانية مساء، في قاعة مبنى الكلية اجتمعت لجنة الحكم على الأطروحة والمكونة من:

د. صادق حسين العويني
د. طارق عبد القادر البشتي
د. عاطف عرفات مسعود

وبعد المداخلة أوصت اللجنة بمنح الباحثة درجة الماجستير في كلية العلوم/ برنامج العلوم الحياتية/ علم الحيوان.

واللجنة إذ تمنحها هذه الدرجة فإنها توصيها بتقوى الله تعالى وزلزل طاعته وأن تسخر علمها في خدمة دينها ووطنه.

وأيضاً ولي التوفيق...

عميد البحث العلمي والدراسات العليا

أ.د. مازن اسماعيل هنية
الموضوع/ استلام النسخة الإلكترونية لرسالة علمية

قام إدارة المكتبات بالجامعة الإسلامية بإستلام النسخة الإلكترونية من رسالة

الطالب/

رقم جامعي: 05320132
قسم: الكيمياء
كلية: العلوم النافعة

وتم الإطلاع عليها، ومطابقتها بالنسخة الورقية للرسالة نفسها، ضمن المحددات المبينة أدناه:

- تم إجراء جميع التعديلات التي طلبتها لجنة المناقشة.
- تم توقيع المشرف/المشرفين على النسخة الورقية لاعتمادها كنسخة معلقة ونهائية.
- تم وضع ختم "عمادة الدراسات العليا" على النسخة الورقية لاعتماد توقيع المشرف/المشرفين.
- وجود جميع فصول الرسالة مجمعة في ملف (PDF) وآخر (WORD).
- وجود فهرس الرسالة، والملخصين باللغتين العربية والإنجليزية بمفاتيح منفصلة (PDF + WORD).
- تطبيق النص في كل صفحة ورقية مع النص في كل صفحة تقابلها في الصفحات الإلكترونية.
- تطبيق التنسيق في جميع الصفحات (نوع وحجم الخط) بين النسخة الورقية والإلكترونية.

ملاحظة: ستقوم إدارة المكتبات بنشر هذه الرسالة كاملة بصيغة (PDF) على موقع المكتبة الإلكتروني.

والله والى التوفيق,

توقيع الطالب
Abstract

**Background:** Cancer is a serious disease that is a burden on individuals and communities. Although many treatments are currently used to treat cancer, they are faced with many obstacles as cancer resistant which reduces the treatment efficacy. Of these cancers, cervical cancer still a leading cause female’s death worldwide. Recently, imidazo[1,2-a]pyridines compounds exhibited a lot of promise to as anticancer agents. Important examples of these compounds are currently at clinical trials.

**Objectives:** This research was conducted to evaluate anticancer activity of newly synthesized imidazo[1,2-a]pyridines (Pico) compounds named 3-[(4-Chlorophenyl)diazemyl]-2,7-dimethylimidazo[1,2-a]pyridine 8 (Pico4) and 3-[(4-Chlorophenyl)diazemyl]-2,5-dimethylimidazo[1,2-a]pyridine 10 (Pico6) against cervical cancer cell line.

**Methodology:** The cytotoxic and anti-proliferative effects of imidazo[1,2-a]pyridines (Pico) compounds were determined against HeLa cell line using MTT assay, trypan blue, scratch assay and Clonogenic assay. Western blot analysis was carried out to detect specific proteins and to determine the mechanism of action of Pico compounds.

**Results:** Our results showed that the imidazo[1,2-a]pyridines (Pico) compounds named Pico4 and Pico6 have a strong anti-growth effect in time and concentration dependent manner in vitro against cervical cancer cell line. The cytotoxic effect of Pico4 against cervical cancer cell line was slightly more potent with IC50 value of 37.8 µM than Pico6 with IC50 value of 42 µM. Trypan blue assay results showed that both compounds induce cell death and anti-proliferative effect in HeLa cell line. Furthermore these compounds induce anti-migration effect where they inhibit cell migration by more than 35% as observed by wound healing assay. Clonogenic survival assay showed that Pico4 reduced HeLa survival rate more than 80% at high cell concentrations. The mechanism by which Pico4 and Pico6 induced cancer cells death by apoptosis as noted by the increasing level of PARP cleavage and increasing levels of P53 in the treated cells.

**Conclusion:** The new imidazo[1,2-a]pyridines (Pico 4 and 6) compounds represent potential active anticancer compounds against cervical cancer cells in vitro.

**Keywords:** Cervical cancer, imidazo[1,2-a]pyridines, biological activities, cytotoxicity, apoptosis
الملخص

خلفية البحث:
السرطان مرضاً خطيراً وهو عبء على الأفراد والمجتمعات. على الرغم من أن العديد من العلاجات تستخدم في الوقت الحالي لعلاج السرطان، إلا أنها تواجه العديد من العقبات التي تقلل من الفعالية حيث ينتج عنها العديد من الآثار الجانبية. لا يزال سرطان عنق الرحم من الأسباب الرئيسية لوفاة الإناث في جميع أنحاء العالم. في الأونة الأخيرة، أظهرت مركبات imidazo[1,2-a]pyridines كعوامل مضادة لسرطان. الأمثلة الهمة من هذه المركبات هي حالياً في التجربة السريرية.

أهداف البحث:
تم إجراء هذا البحث لتقييم النشاطات المضادة للسرطان لسلسلة جديدة من مشتقات imidazo[1,2-a]pyridines تم تصنيعها كمثبطات سرطان عنق الرحم.

منهجية البحث:
تم تحديد التفاعلات السلامة للخلايا لمركبات imidazo[1,2-a]pyridines حيث يتم تطبيق تجربة MTT لتقسيم سمية هذه المركبات على السرطان عنق الرحم. وتم فحص التفاعلات المضادة للسرطان لهذه المركبات من خلال تجربة kill screen فحص عن البروتينات وتحديد الأمثلة التي تعمل بها western blot و試 حا لحاسم  يعذى للعلاق. وتم أيضاً فحص الآلية التي يعمل بها العلاج من خلال تجربة assay مراعاً مركبات.

نتائج البحث:
أظهرت نتائج هذا البحث أن مركبات Pico6 و Pico4 السريني imidazo[1,2-a]pyridines لها تأثير قوي مضاد للنمو معتمدة على الوقت والتركيز في المختبر ضد خلايا سرطان عنق الرحم. وكان التأثير السام للخلايا من Pico4 ضد خلايا سرطان عنق الرحم أكثر فعالية قليلاً مع قيمة IC50 تساوي 42 µM تساوي 37.8 µM مع قيمة Pico6 IC50 تساوي 37.8 µM تساوي 42 µM. أظهرت نتائج اختبار Clonogenic survival أن كلاً من Pico4 و Pico6 قد تكون الألية التي تسبب فيها Clonogenic survival تمكن هجوم الخلايا أكثر من 35% من خلال تجربة wound healing. أظهر اختبار Clonogenic survival أن Pico4 Clonogenic survival أكثر من 80% عند التركيز العالي للخلايا. وقد تكون الآلية التي تسبب فيها Pico6 Clonogenic survival P53 في الخلايا السرطانية في موت الخلايا المبرمج (apoptosis) كما لوحظ من خلال زيادة مستوى P53 في الخلايا المعالجة.

خلاصات البحث:
يمكن لهذه الدراسة أن تكون نقطة بداية لتطوير مجموعات جديدة من مثبطات لعلاج سرطان عنق الرحم.

الكلمات المفتاحية:
سرطان عنق الرحم، السريني، الفعالية البيولوجية، السريني، الموروث المبرمج، imidazo[1,2-a]pyridines.
Dedication

This thesis is dedicated to

My Mother and Father, for their unlimited support from the first step along the way;

My beloved husband, for helping and tolerating me though it all;

To my dearest friends, for their love and engorgement;

And for all of it, I thank Allah for the blessings he gave me and for all of this I have.

To all of you, I dedicate this thesis
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✓ I would first like to thank my thesis advisor Dr. Saeb Aliwaini of the Faculty of Science at The Islamic University of Gaza. The door to Prof. Aliwaini's office was always open whenever I ran into a trouble or had a question about my research or writing. He consistently allowed this paper to be my own work, but steered me in the right direction whenever he thought I needed it.

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✓ I would also like to thank prof. Dr. Adell Awadallah from the department of chemistry at The Islamic university of Gaza for his efforts in targeted compounds synthesis.

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✓ Lastly, I thank every single one who contributed to this research and anyone who helped me in any way no matter how small or big their help was.
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<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ACS</td>
<td>American Cancer Society</td>
</tr>
<tr>
<td>AKT</td>
<td>serine/threonine-specific protein kinase</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bax</td>
<td>Apoptosis regulator</td>
</tr>
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<td>CACO-2</td>
<td>Heterogeneous human epithelial colorectal adenocarcinoma cells</td>
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<td>Caspase-3</td>
<td>Cysteine-aspartic acid protease</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DMSO</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>Ethylene diamin tetracetic acid</td>
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<td>EGFR</td>
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<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>HEK 293</td>
<td>kidney cancer cell</td>
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<td>Human cervical carcinoma cell line</td>
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<tr>
<td>HepG2</td>
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<td>HPV</td>
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<td>IC50</td>
<td>Inhibitory concentration 50%</td>
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<td>IUG</td>
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<tr>
<td>MCF-7</td>
<td>Human cervical cancer cell line</td>
</tr>
<tr>
<td>mM</td>
<td>Milli Moller</td>
</tr>
<tr>
<td>µM</td>
<td>Micro Moller</td>
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<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl(2,5-diphenylte-trazolium bromide</td>
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<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
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<tr>
<td>P21</td>
<td>Cyclin-dependent kinase (CDK) inhibitor</td>
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<td>P53</td>
<td>Tumor suppressor protein</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PCD</td>
<td>Cell Death Programmed</td>
</tr>
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<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<td>PARP</td>
<td>Poly Adp Ribose Polymerase</td>
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<td>PI3K</td>
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Chapter 1
Introduction
Chapter 1
Introduction

Introduction
Cancer is a group of diseases marked by uncontrolled growth and spread of abnormal cells. If the spread is not contained, it can be fatal. Causes can be environmental or lifestyle (tobacco, infectious organisms, chemicals, and radiation) and also can be inherited (mutations, hormones, immune conditions, and mutations that occur from metabolism) (World Health organization, 2014). According to the international agency for research on cancer, around 14 million cases were diagnosed in 2012 worldwide (World Health organization, 2014).

Cancer diagnosis can take up to ten or more years between exposures to external factors (World Health organization, 2014). Staging explain the magnitude or spread of the disease when it is diagnosed. Correct stage determination is crucial in deciding the type of therapy and in assessing prognosis. On the base of the primary tumor’s size, the stage is defined and whether it has spread to other organs of the body (Siegel, Miller, & Jemal, 2016).

The past decade was marked by a breakthrough towards the treatment and realization of the earlier proposed indication of cancer, and with both progress in early diagnosis and in the various treatment modalities, various cancers have become curable (Baskar, Lee, Yeo, & Wei Yeoh, 2012).

Cancer mortality in Palestine was 14% in 2016. Amidst Gaza strip residence cancer incident rate was up to 46.2% among females, and 53.8% among males. Lung cancer was the most cause of cancer deaths in 2016, with 18.6% of deaths related to cancer. Second by colon cancer with 15.1%, then comes brain cancer at 10.4%, and breast cancer by 9.8% then leukemia by 6.6% (Palestinian Ministry of Health, 2017). In 2015, lung cancer was the leading cause of mortality, second by colon cancer. Among females, breast cancer took the first place as the leading cause of, mortality second by colon cancer. The cancer deaths rate was 13.8% of all deaths recorded in Palestine (Palestinian Ministry of Health, 2017).
Cervical cancer ranks the fourth most common cancer and the fourth killing cancer among women all over the world in 2012 (American Cancer Society, 2012). In Gaza strip cervical cancer represents 4.6% of women cancers with a morbidity rate of 21.9 per 100.000 (Palestinian Ministry of Health 2015).

Cervical cancer has two major classes; Squamous Cell Carcinomas (SCCs) which develops in the squamous cells that cover the ectocervix, and Adenocarcinoma which emerges in the gland cells underlay the endocervical canal. Essentially most cases of cervical cancer was caused by constant infection with the human Papilloma virus (HPV) which leads to characterizing cervical cancer by virus-induced illness (Burd, 2003). Other alarming factors include tobacco, the lack of physical activity, pregnancy prevention pills, cervical disease regularly creates from precancerous changes more than 10 to 20 years. About 90% of cervical virulence cases are Squamous Cell Carcinomas, the other 10% are Adenocarcinoma, and a small faction are different sorts. Diagnoses is regularly carried out by cervical screening took after by a biopsy. Medicinal imaging is then done to figure out whether the growth has spread or not (Deverakonda & Gupta, 2016).

The clinical administration of cancer continues to be a challenge for this century. Treatment approach consist of: radiation therapy, surgery, chemotherapy, immunotherapy and hormonal therapy. One of the major treatment of cancer is radiation therapy, roughly 50% of cancer patient undergo radiation therapy during their line of treatment. With 40% rate of cure for cancer. The main objective of radiation therapy is to deprive cancer cells of their multiplication (cell division) capabilities (Baskar et al., 2012). Surgical method in which a surgeon removes tumor, for cancer that can be found in one part of the body, or to partially removal of a tumor, which can help other treatments work better (Siegel, Miller, & Jemal, 2015). Hormone therapy slows down or stops the growth of cancer’s that take advantage of hormones to grow such as prostate and breast cancers. This method prevents hormones production or blocking receptor binding to hormones (Sudhakar, 2009). Chemotherapy is a systemic drug that destroys cancer anywhere it finds in the body. There are various types of chemotherapeutic drugs which are distinguished based on many elements such as its nature, chemical structure, mode of action and its relationship with other medicines (American Cancer Society, 2015b). Side effects for chemotherapy vary in patients, depending on the drug type, and it may also vary from one chemotherapy treatment cycle to the next. Yet, most side effects are
not permanent, and many can be restrained or diminished (Darío Galmarini, Galmarini, & Felipe C. Galmarini, 2012). Side effects comprise feeling exhausted and the lack of energy are the most common. Side effects also include appetite change, and some medicines temporarily change the receptors in tongue buds, alopecia, and some chemotherapy medicines can cause mouth sores and thinking and memory problems following chemotherapy called (chemo brain) (Wade & Kyprianou, 2018).

Cancers have the capability to resist traditional therapies, and the increasing spread of these drugs resistance require more research and treatment improvement (Housman et al., 2014). Cancer drug resistance is a complicated phenomenon that is affected by drug inactivation, drug target alteration, drug efflux, DNA damage repair, cell death inhibition, the epithelial–mesenchymal transition (EMT), inherent cell heterogeneity, epigenetic effects, or any combination of these mechanisms (Zahreddine & Borden, 2013). Therefore we need to develop more effective and safe drugs. Imidazo[1,2-a] pyridine has shown an important biologically anticancer activities. These compounds have been classified as inhibition of cyclin-dependent kinase (CDK), vascular endothelial growth factor receptor (VEGFR), Phosphoinositide 3-kinase (PI3K), the epidermal growth factor receptor (EGFR) (Goel, Luxami, & Paul, 2016). In this regard, this thesis investigates the biological anticancer activity of two new compounds of this family.
1.1 General Objective
To evaluate the effect of two new imidazo[1,2-a]pyridines compounds on cervical cancer cells.

1.2 Specific Objectives
1- The Cytotoxic effects of Pico 4 (3-[(4-Chlorophenyl)diazenyl]-2,7-dimethylimidazo[1,2-a]pyridine 8 ) and Pico 6 (3-[(4-Chlorophenyl)diazenyl]-2,5-dimethylimidazo[1,2-a]pyridine 10 )

2- The Anti-proliferative capacity of Pico 4 (3-[(4-Chlorophenyl)diazenyl]-2,7-dimethylimidazo[1,2-a]pyridine 8 ) and Pico 6 (3-[(4-Chlorophenyl)diazenyl]-2,5-dimethylimidazo[1,2-a]pyridine 10 )

3- The Anti-migration effects of Pico 4 (3-[(4-Chlorophenyl)diazenyl]-2,7-dimethylimidazo[1,2-a]pyridine 8 ) and Pico 6 (3-[(4-Chlorophenyl)diazenyl]-2,5-dimethylimidazo[1,2-a]pyridine 10 )

4- The Anti-survival effects of Pico 4 (3-[(4-Chlorophenyl)diazenyl]-2,7-dimethylimidazo[1,2-a]pyridine 8 ) and Pico 6 (3-[(4-Chlorophenyl)diazenyl]-2,5-dimethylimidazo[1,2-a]pyridine 10 )

5- Determination the mechanism of action by which these two new imidazo[1,2-a]pyridines compounds work.

1.3 Significance:
1. Importance of new drugs to treat important problem such as cancer which kills many peoples around the world.
2. This is the first study to test these two new compounds for anticancer effects.
3. This study provides evidence of how such compounds exerts its anticancer activity.
4. This study, together with other studies on cancer in the Gaza Strip, contributes to raising the level of scientific research on cancer in the Gaza Strip.
Chapter 2

Literature Review
Chapter 2

Literature Review

2.1 Introduction

Cancer develops when normal cells in a particular organ of the body begin to grow out of control. There are diverse types of cancers; in some types cancer cells move to other parts of the body through blood circulation or lymph vessels (metastasis), where they begin to grow. For example, breast cancer cells spread to liver through blood circulation, the cancer is still called as breast cancer, not a liver cancer (World Health Organization., 2017).

In 2015, cancer was the cause for 8.8 million of mortality worldwide (World Health Organization., 2017). As proclaimed by the most recent report of cancer registry unit in Gaza strip, That were followed and documented from 2009 to 2014, there was 7069 cases of cancer that has been reported (The Palestinian Ministry of Health, 2015).

Breast cancer is the most common cancer with 1283 cases in Gaza Strip, which is accounted for 18% of all cancers, with a morbidity rate of 78 per 100.000. Among female’s cancer cases in Gaza, breast cancer comes in the first place with 31.4% of all cancerous diseases (The Palestinian Ministry of Health 2015). Colon cancer was the second common cancer in Gaza strip as stated by the same report, 709 cases was reported with 10% of all cancer cases and morbidity rate of 45 per 100.000. Among Gazans males, colon cancer is the most common with 11.5% of all cancers (The Palestinian Ministry of Health 2015). Anyhow, globally colorectal cancer comes in as the third top cancers among men and the second in women (American Cancer Society, 2015a).

Cervical cancer is the fourth most common cancer and the fourth responsible for cancer deaths among women all over the globe in 2012 (American Cancer Society, 2012). Cervical cancer represents 4.6% of women cancers in Gaza strip with a morbidity rate of 21.9 per 100.000 (The Palestinian Ministry of Health 2015).
2.2 Cervical cancers

Cervix is the lower part of the uterus, the structure that expands during labor to allow the child to pass the birth canal (Soumya D & Arun Kumar R, 2011). Globally, cervical malignancy ranks in fourth place for two things the most normal reason for growth and the most regular reason for death from cancerous disease in women (Deverakonda & Gupta, 2016). In 2012, an expected 528,000 cases of cervical development occurred, with 266,000 mortality. This is about 8% of the total cases and total deaths from cancer. About 70% of cervical development occurs in developing countries. In low-wage countries, it is the most exceedingly recognized cause of disease deaths. The far reaching utilization of cervical screening programs has exceedingly decreased rates of cervical cancer, in developing countries (Gonzalez, 2012). There was 177 cervical cancer cases registered in Gaza Strip, with 4.6% of the total females’ cancerous cases (Global tuberculosis report WHO, 2014).

Cervical cancer usually develops bit by bit over time. Before cancer shows in the cervix, the cells of the cervix go through alterations known as dysplasia, to which abnormal cells begin to emerge in the cervical tissue. Next, cancer cells start to develop and advance more deeply into the cervix and to surrounding organs (Soumya D & Arun Kumar R, 2011). Cervical cancer precursor stage (pre-cancer) lasts several years before developing into invasive stage, in which it provides sufficient opportunities for detection and treatment. After entering cervical epithelial tissues, dangerous HPV contagion intervene with their common functions, which encourage changes specific of pre-cancer (also called dysplasia). (World Health Organization, 2014).
2.2.1 Hela cell line

The HeLa cell line, is the first human cell line cultured for more than 5 decades of culturing study, and it is obtained from an highly aggressive cancer with poor diagnosis, which implies that radiation therapy is insufficient to eliminate the target cells alone (Aziz et al., 2016). In 1951, the pathology department of Johns Hopkins Hospital received Henrietta Lacks’ cervical biopsy for clinical evaluation (Lucey, Nelson-Rees, & Hutchins, 2009). HeLa cells, grew rapidly in cell culture and became the first human cell line used around the world (Beskow, 2016).

2.2.2 Signs and symptoms of cervical cancer

Almost all cervical cancers starts in the cells in the transformation zone. These cells dose not all of a sudden turn into cancer. Rather, slowly the normal cells of the cervix first develop pre-cancerous changes that shift into cancer (American Cancer Society, 2016). Symptoms are conceived in the early stages of this cancer; when symptoms are sitting, they can comprise of

2.2.3 Risk factors
The fundamental malady of cervical precancerous and squamous cervical cancer is determined with chronic infection with one or more of the dangerous (cancer-causing or oncogenic) types of human papillomavirus (World Health Organization, 2014). (HPV) contagion is the major risk factor for the evolving of the disease (Deverakonda & Gupta, 2016). The virus advances mainly through intimate skin-to-skin contact and is generally asymptomatic. As a matter of fact, it is crucial to understand that HPV infections are popular in healthy women and are typically cleared efficiently by the immune system; the infection hardly does stick and turn into in cervical cancer (Saslow et al., 2013). HPV infection Permanence and its development to cancer can be attributed to many factors, including immunosuppression, high parity (number of childbirths), and cigarette smoking. Everlasting use of birth control pills is also associated with increased exposure of cervical cancer. (American Cancer Society, 2018).

2.2.4 Types of cervical cancer
The classification of cervical cancers and cervical pre-cancers is determined by how they look under a microscope.

I. **Squamous cell carcinomas**: Squamous cell carcinomas generally begin in the transmutation zone (where the exocervix joins the endocervix). These cancers grow from cells in the exocervix and the cancer cells have characteristics of squamous cells under the microscope (American Cancer Society, 2016).

II. **Adenocarcinomas**: Cervical adenocarcinoma grows from the mucus-producing gland cells of the endocervix. Cervical adenocarcinomas has widespread in the past 20 to 30 years.

III. **Adenosquamous carcinomas**: also called mixed carcinomas. Uncommon, cervical cancers which have features of both squamous cell carcinomas and adenocarcinomas.

2.2.5 Cervical cancer treatment
Treating cervical cancer can be done through several mechanisms, and the best treatment is chosen according to many criteria, which include type and stage of cancer, possible complications, the patient choice and his general health status (Cancer.Net, 2017). The
appropriate medication, with the help of professionals and the suitable technology can provide crucial life support to cancer patients. Nonetheless, this circumstance also means cervical cancer patients should go through a long and complex process. The major kinds of cervical cancer therapies include surgery, chemotherapy, radiation therapy, and Targeted therapy. Some patients with cervical cancer will have a combination of therapies, for instance surgery with chemotherapy and/or radiation therapy (American Cancer Society, 2016a). The next section of this chapter will provide a general overview of the key cervical cancer therapy types.

2.2.5.1 Surgery is a medicinal procedure to check, eliminate or mend tissue. It can be used to cut out considerable amounts of tissue or an entire organ in order to lower risk of developing cervical cancer. The surgical procedure is divided into the four major steps: Cryosurgery, Laser surgery, Conization and Hysterectomy. With Cryosurgery a metal probe cooled down with liquid nitrogen then placed directly on the cervix. This procedure kills the abnormal cells by freezing. It can be operated in a doctor’s office or medical center. After cryosurgery, patient may experience a lot of watery brown discharge for several days. Cryosurgery is used to cure carcinoma in situ of the cervix (stage 0), and not for invasive cancer (Rydzewska, Tierney, Vale, & Symonds, 2010). Laser surgery include a focused laser beam, aimed through the vagina, and is used to burn off abnormal cells or to cut out a small piece of tissue for examination. It can be operated in a doctor’s office or medical center while the patient is under local anesthesia. Laser surgery is used to cure carcinoma in situ of the cervix (stage 0). It cannot be used to treat invasive cancer (Rydzewska et al., 2010). With Conization a coned piece of tissue is removed from the cervix. Using a surgical or laser knife (Rydzewska et al., 2010). Hysterectomy is the surgical removal of the uterus but not the structures next to it. The vagina and pelvic lymph nodes are not removed also. The ovaries and fallopian tubes are mostly left in place unless there is a reason to remove them (American Cancer Society, 2016). After surgery, most patients will suffer from pain in the operated part and contamination is another issue that can occur after surgery (American Cancer Society, 2016d). Other risk factors of surgery comprise of bleeding, damage to neighboring tissues, drug effects and dysfunction in some body functions (National Cancer Institute, 2015).
2.2.5.2 **Radiation therapy** (also known as radiotherapy) is a cancer therapeutic procedure that uses high dosages of radiation to wipe out cancer cells and diminish tumors. In low dosages, radiation is applied as an x-ray to look internal organs and take pictures, such as x-rays of your teeth or broken bones (American Cancer Society, 2016d). Radiation therapy is a fundamental modality of cancer offered in different dosages to treat cancer or lessen symptoms (Aggarwal et al., 2018).

Radiation therapy can be used solely to treat some cervical cancer used with other cancer therapeutics, during, or after surgery or chemotherapy (Delaney, Jacob, Featherstone, & Barton, 2005).

**Types of Radiation therapy**

i. **External radiation therapy (or external beam radiation):**
   A topical therapy, which means that the radiation addresses a specific part of the body by directs radiation on the tumor area from all directions (American Cancer Society, 2016d).

ii. **Internal radiation therapy (Brachytherapy):**
   A source of radiation is put inside the body. One type of internal radiation treatment is called brachytherapy. In brachytherapy, a radioactive implant is planted in the body, in or next to the cancer, used to cure various types of cancers (American Cancer Society, 2016d).

Radiotherapy by itself, in some stages of cervical cancer is the best option while in other cases it is preferred to have surgical procedure followed by radiotherapy and for additional stages it's better to administer chemotherapy and radiotherapy with one another, researches have demonstrated that concomitant chemo-radiation increases the survival for patients with cervical cancer and its toxicity to normal cells is tolerable but its long- term toxicity has not been determined yet (Cancer.Net, 2016a). Some studies have proposed that raising the temperature of the tumor with radiation benefits patients to live longer and also prevents the cancer recurrence, this procedure is called hyperthermia (ACS, 2016c). Women who radiation therapy will considerable side effects, for instance killing of adjacent normal cells, fatigue, skin rashes, fever or chills, mouth sores, queasiness and diarrhea (American Cancer Society, 2016d).
2.2.5.3 Chemotherapy is a medicinal term expressed to depict chemical agents used in cancer treatment. Distinct from surgery and radiation, chemotherapy is applied as a systemic mean to cure cancer and is particularly significant for patients with advanced stages of cancer. At the present time, up to 100 chemotherapeutic agents are applied either as single therapy or in combination with other therapies and categorized based on several key factors such as the course of its work, chemical structure and its interaction with other medicines (American Cancer Society, 2016b).

Cisplatin, Topotecan, Carboplatin, Gemcitabine and Paclitaxel are the most used chemo drugs for curing cervical cancer, few of them are often combined. Some drugs are used to a minor extent as 5-fluorouracil (5-FU), mitomycin, docetaxel, irinotecan and ifosfamide (ACS, 2016b). Until now, cisplatin was the first choice drug for metastatic cervical cancer but it has been reported that doublets, triplets, and quartets gives a higher response rate than single-cisplatin (Long, 2007). The massive problem facing chemical drugs is, while they destroy cancer cells they also destroy some normal cells along the way, which give a rise to side effects. For instance, if chemotherapy kills normal hematopoietic cells in the bone marrow it may result in increasing the risk of infection, serious hemorrhage after minor injuries and heavy breathing. But Common side effects of chemotherapy includes vomiting, hair loss, mouth sores, loss of appetite and fatigue (ACS, 2016b). And kidney damage (Cancer.Net, 2017).

Nearly all of the existing drugs have limited efficacy against cancers, to some extent because of, tumour cells gaining resistance to chemotherapy (Sawicka, Kalinowska, Skierski, & Lewandowski, 2004). In numerous cases, cancer cells alter the expression of cell surface receptors decreasing the drug intake and boosting the drug efflux. Regarding enzyme-inhibiting chemotherapies, drug resistance can emerge from genetic mutation or by gene amplification of the intended enzymes (Gottesman, 2002). Resistance of tumour cells to DNA damaging agents are mostly linked with modifications in the DNA damage response to maximize the DNA repair ability (D. Wang & Lippard, 2005). particular combination of treatments can be applied to boost the influence of a certain chemotherapy and to reduce drug resistance (Cheung-Ong, Giaever, & Nislow, 2013). Nevertheless conventional chemotherapeutic agents are general, cytotoxic, strong and causes unwanted side effects, it doesn’t only kill rapidly growing cancer cells, but also kills or slows the growth of healthy cells that typically grow and divide rapidly like blood-generating
cells in the bone marrow, mouth cells, digestive tract, reproductive organs and hair follicles. For instance, using doxorubicin and other Anthracyclines have been associated with cardiac toxicity, including cardiomyopathy and congestive heart failure (CHF). Harming healthy cells can be the cause of side effects, like fatigue, hair loss, infection, anemia, nausea, vomiting, appetite changes, nerve and muscle problems, urine and bladder changes and kidney problems, weight changes and fertility problems (American Cancer Society, 2016c; Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004).

As a result of these side effects for general traditional chemotherapy, scientists engineered new drugs that kill cancer cells more precisely than traditional chemotherapy and have fewer side effects. These drugs named targeted therapies.

### 2.2.5.4 Targeted therapy

Targeted therapy aims to treat certain traits in cancer cells that distinguishes them from normal cells (Vijaya, 2015). Cancer cells make some transformation on their own genomes to allow the continuous divide forming tumors, attack neighboring tissues and migrate to different organs of the body. These genetic alterations that occur in cancer cells manifest as overexpression or down regulation of certain molecules or signals that responsible for the keeping of the carcinogenesis process (American Cancer Society, 2013). Targeted therapy stops the growth, development and spread of cancer cells without damaging normal cells, that’s why its conceder to be the best treatment among other conventional types in reducing side effects (American Cancer Society, 2013). Various possible targeted drugs are being examined in phase I/II clinical trials to detect molecular targets and therapeutic mechanisms in cervix. For instance, targeted drug bevacizumab (Avastin) is one of the angiogenesis inhibitors that targets vascular endothelial growth factor (VEGF) which aid cancer cells to create blood vessels in a process called angiogenesis in the advanced stages of cervical cancer. Cetuximab is another drug that targets epidermal growth factor receptor (EGFR) that plays crucial role in tumor growth (ACS, 2016d.; del Campo, Prat, Gil-Moreno, Pérez, & Parera, 2008). Another drug, pazopanib acts by blocking certain growth factors that help cancer cells survival (Tsuda, Watari, & Ushijima, 2016). Unfortunately, while most of the current therapies still show limited success in cancer treatment, they cause severe undesirable side effects. Therefore more efforts are invested to find more effective and safe therapies. In this regard the Imidazo[1,2-a]pyridines recently showed a promise as new anticancer agents.
2.3 Imidazo[1,2-a]pyridines

A category of compounds that have sparked interest in the pharmaceutical chemistry field are imidazo[1,2-a]pyridines (Figure 2.2). Fully aromatic ring system with electrons on nitrogen at position 4 to complete the aromaticity. They display an important biological activity, extremely remarkable as enzyme inhibitors, receptor ligands and anti-infective agents (Gueiffier, Mavel, Lhassani, Elhakmaoui et al., 1998; Dömling & Ugi, 2000).

(Figure 2.2) – The imidazo[1,2-a]pyridine ring and its numbering

Several compounds of this family showed considerable biological activity and some imidazo[1,2-a]pyridines turned into commercially available drugs. Of these drugs; olpirone for cardiovascular issues, zolpidem for insomnia, alpidem as anxiolytic, miroprofen as a painkiller and zolmidine for ulcer treatment. (Zhuang, Kung, Wilson, Lee, Plossl, Hou, Holtzman & Kung, 1998). Importantly, till now there is no any anticancer drug derived from this family. However, there are several lines of evidence that Imidazo[1,2-a]pyridines might lead to improvement of anticancer strategies and could be effective anticancer agents (Kishino, Moriya, Sakuraba, Sakamoto, et al., 2009). The following sections focuses on the most common biological effects of these compounds.
2.3.1 Inhibition of Centromere Protein E

Centromere protein E (CENP-E) is an important protein to organize the cell-division cycle and for movement from metaphase to anaphase during mitosis (Sardar & Gilbert, 2012). To block this protein, a specific potent inhibitor of human CENPE has been synthesized. The formula of this compound is (3-chloro-N-{(1S)-2-[(N,N-dimethylglycyl)amino]- 1-[(4-8-[(1S)-1-hydroxyethyl]imidazo[1,2-a]pyridin-2-yl]phenyl)methyl}ethyl)- 4-[(1-methylethyl)oxy] benzamide and also called (GSK923295). The inhibitory effect of this compound was very potent with an IC\textsubscript{50} of 0.003 μM. GSK923295 also inhibited the growth and cell proliferation of human tumors in vivo and cancer cell lines by inducing cell cycle arrest and apoptosis. The compound showed all features of a clinical drug, including solubility profile and therefore, this drug is also under human clinical trials (Qian, McDonald, Zhou, Adams, Parrish, et al., 2010). GSK923295 was recognized as an allosteric inhibitor of CENP-E kinesin motor ATPase activity. GSK923295 blocked the release of inorganic phosphate that stabilized the CENP-E motor domain fundamental interaction with microtubules. Treating 237 cell lines by GSK923295 produced a growth inhibitory activity (GI50) in the range of 0.012 μM to 10 μM, with an average GI50 of 0.253 μM. In addition, it was administered to mice with colon cancer and produced an increase in multitude of mitotic cell cycle arrest and apoptotic bodies in tumors (Wood, Lad, Luo, Qian, Knight, Nevins, Brejc et al., 2010).

2.3.2 Inhibition of Nicotinamide Phosphoribosyl Transferase

Imidazo[1,2-a]pyridines with amide motifs were synthesized as novel inhibitors of nicotinamide phosphoribosyl transferase (Nampt) (Zheng, Bauer, Baumeister, Buckmelter et al., 2013). One compound of these compounds exerted inhibitory action against Nampt and A2780 cell lines with IC\textsubscript{50} amounts of 0.005 μM and 0.002 μM respectively. Furthermore, it showed potent antitumor effects on many cancer cell lines such as; glioblastoma (U251, IC\textsubscript{50} = 0.0018 μM), fibrosarcoma (HT1080, IC\textsubscript{50} = 0.0021 μM), prostate cancer (PC3, IC\textsubscript{50} = 0.0027 μM), pancreatic cancer (MiaPaCa2, IC\textsubscript{50} = 0.0074 μM) and colon cancer (HCT-116, IC\textsubscript{50} = 0.002 μM) on mice xenografts. Plasma clearance and volume of distribution were noted to be 52.4 mL/min/kg and 6.7 L/kg with intravenous route at a dose of 5 mg/kg of 6-Substituted imidazo[1,2-a]pyridine. Obvious tumor regression was observed with this compound at a dose of...
50 mg/kg QD and U251 human glioblastoma multiforme tumor xenograft model for 7 days with no loss of body weight.

2.3.3 Inhibition of tubulin organization

A study reported series of novel 2-aryl-3-arylamino-imidazo-pyridines/pyrazines were synthesized. These compounds were tested on HEK-293T kidney, MCF-7 breast, and HeLa cervical cancer cell lines. HEK293T cells treated with novel 2-aryl-3-arylamino-imidazo-pyridines/pyrazines compounds for anti-tubulin activity showed IC50 of 5 μM and MCF-7 cells showed IC50 of 12 μM and HeLa cells showed IC50 of 10 μM. It has been noted that clonogenic cell survival of HEK293T cells was significantly lowered on treatment with novel 2-aryl-3-arylamino-imidazo-pyridines/pyrazines compounds. Also showed significant dependent anti-cell migration potential in HEK 293T cells (Sanghai, Jain, Preet, Kandekar et al., 2014).

Cell cycle profile was investigated in this study on HEK 293T cells and it was noted that novel 2-aryl-3-arylamino-imidazo-pyridines/pyrazines compounds induced cell cycle arrest in the G2/M phase by inhibition of caspase 3 (Sanghai, Jain, Preet, Kandekar et al., 2014).

Investigation of the apoptotic effect of these compounds was performed on HEK 293T cells and showed that the expression level of apoptotic marker proteins have significant increase. This was obvious for p53 expression and cleaved PARP. Furthermore the activation of caspase-3 induced apoptosis in HEK 293T cells.

These compounds also prevented polymerization of tubulin and also disrupted the tubulin microtubule complex. This perhaps because of interaction of compounds at the, β-tubulin heterodimer interface with the colchicine bonding site (Sanghai, Jain, Preet, Kandekar et al., 2014).

2.3.4 Inhibition of Topoisomerase-II

A study reported the effect on Topoisomerase-II activity by imidazo[1,2-a]pyridine. It showed that 4-(3-(Tert-butylamino)imidazo[1,2-a]pyridin-2-yl)-benzoic acid displayed potent cytotoxic effects on HEK293 kidney cancer cells with (IC50 = 12 μM) and breast cancer cells MCF-7 with (IC50 = 15 μM) while it has less cytotoxic effect on MCF 10A normal breast cells. The results from DAPI assay reveal that 4-(3-(Tert-butylamino)imidazo[1,2-a]pyridin-2-yl)-benzoic acid induced apoptosis in HEK293 cells. The percent of apoptosis increased (20, 40, and
80) with increasing of compound concentration 15, 25 and 35 μM respectively. The data showed that the levels of apoptotic protein markers (Bax, Caspase-3 and cleaved PARP proteins) in treated HEK293 cells increased with the increment of the concentration from 0 to 25 μM on the other hand, anti-apoptotic protein BCL-XL level reduced. Cell cycle profile of treated HEK293 cells which analyzed by fluorescence activated cell sorter (FACS) exhibited that this compound induces apoptosis (Sub G0) in a dose dependent manner in G1/S boundary phase of cell cycle (Baviskar, Madaan, Preet, Mohapatra et al., 2011).

2.3.5 Inhibition of Wnt/β-Catenin signaling

Wnt/β-catenin signaling plays a significant role in controlling proliferation and apoptosis (Zhang et al., 2015). Imidazo[1,2-a]pyridines worked as inhibitors of Wnt/β-catenin signaling. Releasing of Wnt/β-catenin signaling gives rise to cancer. Verifying the effect of compounds N-Benzyl-4-(6-methylimidazo[1,2-a]pyridin-2-yl)aniline and N,N-Dibenzyl-4-(7-methylimidazo[1,2-a]pyridin-2-yl)aniline on Wnt/β-catenin signaling. N-Benzyl-4-(6-methylimidazo[1,2-a]pyridin-2-yl)aniline with (IC50 = 11.1 ± 0.005 μM) and N,N-Dibenzyl-4-(7-methylimidazo[1,2-a]pyridin-2-yl)aniline with (IC50 = 24.1 ± 0.02 μM) showed great activity HT-29 cell line (Cosimelli, Laneri, Ostacolo, Sacchi et al., 2014). These compounds showed potent antitumor effects on human cancer cell lines, HT-29, LoVo with mutated APC gene, HepG2 with endogenic mutated gene of β-catenin and A549 with high levels of Wnt2. N-Benzyl-4-(6-methylimidazo[1,2-a]pyridin-2-yl)aniline (GI50 = 8.8 ± 0.4 μM, 42.6 ± 3.5 μM) and N,N-Dibenzyl-4-(7-methylimidazo[1,2-a]pyridin-2-yl)aniline (GI50 = 6.9 ± 1.2 μM, 5.7 ± 0.5 μM) displayed great GI50 amounts towards HT-29 and LoVo cells respectively. N-Benzyl-4-(6-methylimidazo[1,2-a]pyridin-2-yl)aniline displayed no inhibition opposed to hepG2 (>100 μM) and higher GI50 value in A549 cells (56.1 ± 11.2 μM). N,N-Dibenzyl-4-(7-methylimidazo[1,2-a]pyridin-2-yl)aniline displayed greater activity against HepG2 (18.4 ± 10.0 μM) and A549 (17.2 ± 6.9 μM) cancer cell lines. N-Benzyl-4-(6-methylimidazo[1,2-a]pyridin-2-yl)aniline and N,N-Dibenzyl-4-(7-methylimidazo[1,2-a]pyridin-2-yl)aniline treatment with activated Wnt target genes, especially c-myc and cyclin D1, decreased the proliferation zone (Cosimelli, Laneri, Ostacolo, Sacchi et al., 2014). They were ineffectual against normal cells, denoting their selectivity for cancer cells through deregulation of the Wnt pathway. These compounds also acted as Wnt
inhibitors in in vivo zebra fish models by lowering the TCF/LEF transcription in the intestinal zone (Cosimelli, Laneri, Ostacolo, Sacchi et al., 2014).

2.3.6 Inhibition of Phosphoinositide-3-Kinase (PI3K) and Akt

In cancer growth, angiogenesis is a key player because solid tumors need a blood supply for invasion and metastasis (Kim, Khursigara, Sun, Franke, & Chao, 2001). The PI3K/Akt pathway performs an important role in angiogenesis, and inhibiting these pathway leads to antiangiogenic activity towards cancer cell lines. The pyridyl sulfonamide moiety well-known to inhibit PI3Kα at 10 μM concentricity. Ethyl 6-(5-(phenylsulfonamido) pyridin-3-yl)imidazo[1,2-a]pyridine-3-carboxylate named (HS-173) has showed effective inhibiting activity towards PI3Kα with IC50 value of 0.0008 μM, by working as a competitive inhibitor. It inhibits the cancer cells of liver and breast at concentrations from 0.1 to 10 μM. It prompts the suppression of Akt phosphorylation, mTOR and downstream factors such as p70S6K and GSK3β in Hep3B cells, indicate the complete deactivation of the PI3k pathway. (Lee, Jung, Jeong, Hong et al., 2013).

More recent study showed a potent anti-cancer activity of N-(5-(3-(5- methyl-1,2,4-oxadiazol-3-yl)imidazo[1,2-a]pyridin-6-yl)pyridin- 3-yl)benzenesulfonamide (HS-104) against human breast cancer cell lines. HS-104 displayed effective inhibitory activity against PI3Kα, with IC50 < 0.01μM. It blocked the PI3K-Akt-mTOR pathway in breast cancer cells by restraining the Akt phosphorylation, mTOR and downstream factors such as GSK3β, p70S6K and 4E-BP1 in MCF-7, T47D and SkBr3 breast cancer cell lines. HS-104 showed anti-proliferative activity (IC50) against breast cancer cell lines MCF-7 (1.2 μM), T47D (1.1 μM) and SkBr3 (4.8 μM) in a dose dependent manner. HS-104 (5 μM) induced apoptosis in breast cancer cells MCF-7, T47D and SkBr3 to 25%, 40% and 38% respectively. To investigate the inhibitory effect of HS-104 on tumor development in vivo, SkBr3 mouse xenograft models were used and after 24 days of HS-104 treatment, average tumor volume and tumor weight in mice were reduced by 50% and 60% respectively in comparison to control mice. Low toxicity of HS-104 was confirmed by its administration in tumor bearing mice (Jung, Zheng, Jeong, Choi et al., 2013).
2.3.7 Inhibition of cell proliferation by Akt-mediated regulation

A study reported the synthesized of a novel series of selenylated imidazo[1,2-a]pyridines, 7-methyl-3-(naphthalene-1-ylselanyl)-2-phenylimidazo[1,2-a]pyridine, named IP-Se-05, and 3-((2-methoxyphenyl)selanyl)-7-methyl-2-phenylimidazo[1,2-a]pyridine, named IP-Se-06 against MCF-7, HeLa and HRT-18 cancer cell lines (Almeida et al., 2018). Both compounds IP-Se-05 and IP-Se-6 showed high cytotoxicity for MCF-7 cells with IC50 26.0 μM and 12.5 μM, respectively and Hela cervical cell line with IC50 29.9 μM and 7.4 μM, respectively and HRT-18 colorectal cell line with IC50 18.8 μM and 31.3 μM, respectively. Additionally clonogenic cell survival of IP-Se-05 and IP-Se-6 compounds showed a high ability to inhibit MCF-7 proliferation in non-toxic concentration (15.6 μM and 7.5 μM, respectively). IP-Se-06, which inhibited cell growth by 90% compared to untreated cells (Almeida et al., 2018). The antitumor activities of IP-Se-05 and IP-Se-6 compounds were related to blocking the PI3K/Akt/mTOR, a pathway closely associated with the development, progression and metastases of breast cancer. Furthermore, it showed that IP-Se-05 and IP-Se-6 compounds can be cyclin-dependent kinase inhibitors for tumor cells. Furthermore, IP-Se-05 and IP-Se-06 with CT-DNA interaction was studied using absorption titration and fluorescence spectroscopy. At a fixed concentration of CT-DNA (150 mM) and by increasing concentrations of the IP-Se-05 and IP-Se-06, the absorbance bands in the UV region exhibit a hypochromism, thereby suggesting that IP-Se-05 and IP-Se-06 were binding to DNA by intercalation.

The Comet assay estimated the effect of IP-Se-05 and IP-Se-06 on DNA of MCF-7 cells. Cancerous cells with IP-Se-05 (15.6 mM) and IP-Se-06 (7.5 mM) was treated for 72 h had a significant DNA damage (p < 0.001) compared with untreated cells (Almeida et al., 2018).
Chapter 3

Material and methods
Chapter 3
Material and methods

3.1 Materials

3.1.1 Chemicals and Reagents

The chemicals and reagents used in this study are summarized in Table 3.1.

Table (3.1): Chemicals and reagents were used in this study.

<table>
<thead>
<tr>
<th>#</th>
<th>Reagents</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMEM– Dulbecco's Modified Eagle Medium</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>2</td>
<td>RPMI1640-Roswell Park Memorial Institute medium media</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>3</td>
<td>Trypsin</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>4</td>
<td>Phosphate Buffer Saline</td>
<td>AppliChem- Germany</td>
</tr>
<tr>
<td>5</td>
<td>Fetal Bovine Serum</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>6</td>
<td>Primary Antibody</td>
<td>Santa cruzUSA</td>
</tr>
<tr>
<td>7</td>
<td>Secondary Antibody</td>
<td>Santa cruzUSA</td>
</tr>
<tr>
<td>8</td>
<td>MTT Kit</td>
<td>Sigma USA</td>
</tr>
<tr>
<td></td>
<td>Trypan Blue</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>9</td>
<td>Western Blot Reagents</td>
<td>AppliChem- Germany</td>
</tr>
<tr>
<td>10</td>
<td>Ethylene diamine tetra etic acid (EDTA)</td>
<td>AppliChem- Germany</td>
</tr>
<tr>
<td>11</td>
<td>Dimethyl sulfoxide(DMSO)</td>
<td>AppliChem- Germany</td>
</tr>
<tr>
<td>12</td>
<td>(A) 3-[(4-Chlorophenyl)diazynyl]-2,7-dimethylimidazo[1,2-a]pyridine 8 (Pico4).</td>
<td>The compounds are provided by prof. Dr. Adell Awadallah from department of chemistry – The Islamic university of Gaza</td>
</tr>
<tr>
<td></td>
<td>(B) 3-[(4-Chlorophenyl)diazynyl]-2,5-dimethylimidazo[1,2-a]pyridine 10 (Pico6).</td>
<td></td>
</tr>
</tbody>
</table>
3.1.2 Disposables

Table (3.2): Major Disposables used in this study.

<table>
<thead>
<tr>
<th>#</th>
<th>Item</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Culture Flask</td>
<td>Intron</td>
</tr>
<tr>
<td>2</td>
<td>96 well plate</td>
<td>Intron</td>
</tr>
<tr>
<td>3</td>
<td>24 well plate</td>
<td>Intron</td>
</tr>
<tr>
<td>4</td>
<td>6 well plate</td>
<td>Intron</td>
</tr>
<tr>
<td>5</td>
<td>Petri dish (6cm)</td>
<td>Intron</td>
</tr>
<tr>
<td>6</td>
<td>Pipettes</td>
<td>Intron</td>
</tr>
<tr>
<td>7</td>
<td>Falcon Centrifuge Tube</td>
<td>Intron</td>
</tr>
</tbody>
</table>

3.1.3 Equipment

Table (3.3): Major Equipment’s used in this study.

<table>
<thead>
<tr>
<th>#</th>
<th>Instrument</th>
<th>Type</th>
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</thead>
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<td>Centrifuge</td>
<td>Centurion scientific Ltd</td>
</tr>
<tr>
<td>2</td>
<td>Safety cabinet</td>
<td>Biological Safety Cabinet (NB-602 WS/WSL)</td>
</tr>
<tr>
<td>3</td>
<td>Western Blot Unit</td>
<td>BIO RAD Mini- protean @ tetra system</td>
</tr>
<tr>
<td>4</td>
<td>Micropipettes</td>
<td>Scilogex (100-1000µM)</td>
</tr>
<tr>
<td>5</td>
<td>Dispenser</td>
<td>JENCONS SELPETTE</td>
</tr>
<tr>
<td>6</td>
<td>CO2 Incubator</td>
<td>Nb-203xl</td>
</tr>
<tr>
<td>7</td>
<td>Elisa Reader</td>
<td>VMax® Kinetic ELISA Microplate Reader with Softmax® Pro Software</td>
</tr>
<tr>
<td>8</td>
<td>Inverted microscope</td>
<td>Motic- at3ie- tension</td>
</tr>
</tbody>
</table>

3.1.4 Cell lines

Table (3.4): Cell lines used in this study.

<table>
<thead>
<tr>
<th>#</th>
<th>Cell line</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>3-</td>
<td>Uterine Cervical adenocarcinoma (HeLa)</td>
<td>Dr Johnny Stiban, Birzeit University of Palestine</td>
</tr>
</tbody>
</table>
3.2 Methods

3.2.1 Study design

An Experimental in vitro study.

3.2.2 Setting

Labs of Biology and Biotechnology department at the Islamic University of Gaza.

3.2.3 Cell culture

HeLa (Cervical cancer cells) were maintained in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37°C in a 5% CO2 and 95% air-humidified incubator. Media was replaced every 2-3 days (Aliwaini et al., 2015; H. C. Wang et al., 2016).

3.2.4 Treatments

A panel of compounds belong to imidazo[1,2-a]pyridines (Picos) was synthesized by Prof. Adel M. Awadallah and his colleagues which includes 3-{(4-Chlorophenyl)diazenyl}-2,7-dimethylimidazo[1,2-a]pyridine 8 (Pico4) and 3-{(4-Chlorophenyl)diazenyl}-2,5-dimethylimidazo[1,2-a]pyridine 10 (Pico6) (Figure 3.1). Two compounds Pico4 and Pico6 were selected for solubility issues. The compounds pico4 and pico6 used in this study were dissolved in in heated DMSO (up to 100°C) to enhance their solubility to give a final concentration of 10 mM stored at room temperature for no more than 7 days. In order to get the final concentration, subsequent dilutions in the appropriate media for cell line were prepared. Vehicle treated cells were incubated in normal media with DMSO (the vehicle in which Pico-compounds was dissolved in) as shown in (Figure 3.2).
Figure (3.1): Structural formulas of the pico compounds

(a) 3-[(4-Chlorophenyl)diazenyl]-2,7-dimethylimidazo[1,2-a]pyridine 8 (Pico4)
(b) 3-[(4-Chlorophenyl)diazenyl]-2,5-dimethylimidazo[1,2-a]pyridine 10 (Pico6)

Figure (3.2): Dilutions of the pico compounds in the appropriate media to get the final concentration.
3.2.5 Cell morphology

Cells were plated at suitable numbers in order to obtain 60-70% confluency on the day of treatment. After treating the cells with the (Picos) compounds the morphological changes were monitored and photographed using an inverted light microscope (Olympus 1X71, USA) and camera (Zeiss AxioCam, Germany) respectively. Any morphological changes were photographed using a light microscope.

3.2.6 Cytotoxicity assays (MTT)

To determine the cytotoxic effect of the indicated compound HeLa cells were seeded respectively (6000 HELA cells/well), in quadruplicate in a 96-well plate and treated after 24 hours with a range of the indicated concentrations of specific compounds or vehicles for 48 hours. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and it is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells. Briefly, 10μL of MTT solution was added to each well and incubated for 4 hours at 37°C. This was followed by the addition of 100μL solubilization buffer (10% SDS in 0.01 M HCl) and incubated overnight at 37°C. Absorbance (550 nm) was then determined for each well and the mean cell viability was calculated as a percentage of the control. Two separate experiments were performed to determine the concentration of (pico) compounds required to kill 50% of the cells (IC\textsubscript{50}). The IC\textsubscript{50} values were calculated from linear equation from Microsoft (Aliwaini et al., 2015).

3.2.7 Viability assay /trypan blue

To determine the ability of (pico) compounds to induce cell death in cancer cells, HeLa cells were seeded respectively (600,000 HELA cells/well), in duplicate in a 6-well plate and treated in a second day with the indicated concentrations of specific compounds or vehicles. Cell viability was assessed by the trypan blue assay after 24 and 48 hours of treatment. And total cells and viable cells were counted by trypan blue exclusion as follows. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue dye, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will
have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Floating and adherent cells were collected at each time point, centrifuged, and resuspended in medium. The cells were then diluted at a 1:9 ratio of 0.4% trypan blue (Sigma) and scored under a light microscope. Viable (unstained) and nonviable (blue-stained) cells were counted, and the total numbers of living and dead cells were calculated (Yamasaki et al., 2008).

### 3.2.8 In vitro cell migration assay

In vitro scratch motility assay is based on the observation that, upon creation of a new artificial gap, so called “scratch”, on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the “scratch” until new cell–cell contacts are established again. Cells were grown to confluence in 6cm tissue culture dishes. A linear wound was made by scratching through the monolayer using a sterile 100 µl pipette tip. To remove cell debris, the growth medium was replaced and several markings were made along the edges of the scratch line which were used as reference points and the wound widths measured at the time of the scratching (0 hour) and thereafter at 24, 48 and 72 hours intervals. Pictures were taken using a phase contrast microscope and migration distances were measured using Axiovert software (Zeiss, Germany). The difference in width represents the distance migrated in μm (Liu et al., 2016).

### 3.2.9 Clonogenic survival assay

The ability to generate clones is interpreted as a trait of aggressive tumor cells that harbor tumor-initiating capabilities. Cells were pre-cultured and treated with 40μM of Pico-compounds for 24 hours, re-plated at low density (100,300,500 and1000) and incubated for 14 days. Following 14 days of growth, surviving cells were fixed in methanol for 3 min at RT and stained with 10% Giemsa stain (Sigma, USA) in deionized water for 20 min at RT. Stained colonies were washed 3 times in 1X PBS. Percentage change in surviving colonies is calculated by dividing the number of colonies calculated in the sample by the number of colonies calculated in the control multiplied by 100% (Aliwaini et al., 2015).
3.2.10 Western blot analysis

For SDS-PAGE and immunoblotting, cells were plated at $6 \times 10^5$ in 6 cm dishes and treated with 40µM Pico-compounds for 24 and 48 hours. Cells were analyzed in whole cell lysis buffer (0.5 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% b-mercaptoethanol and 0.02% bromophenol blue) and samples boiled for 10 min. Proteins were resolved by SDS/PAGE (8–15% gels) as required and transferred to Hybond ECL membranes (Amersham Biosciences). The membranes were incubated with primary antibodies against p53 (sc-126), p38 (M0800) and PARP1/2 (sc-7150), (Santa Cruz, CA, USA). After the primary antibody incubation, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (1:5000) (Biorad) and antibody-reactive proteins were visualized using the chemiluminescence reaction (ECL) detection system (Thermo Scientific, Hudson, NH, USA) (Aliwaini, Swarts, Blanckenberg, Mapolie, & Prince, 2013; Yamasaki et al., 2008).

3.2.11 Statistical analysis

Data presented are mean ± SEM (Standard error of the means) of three independent experiments. Statistical significance was assessed between the groups using the Student’s t-test. A value of $P < 0.05$ was accepted as statistically significant.
Chapter 4

Results
Chapter Four

Results

Cancer is a main public health issue worldwide and it records annually for one in seven deaths (American Cancer Society, 2012). It occurs due to uncontrolled cell division with the ability of these cells to penetrate and migrate to different parts of the human body (World Health Organization., 2017). Breast, colorectal, lung, cervix and stomach cancers are the most frequent cancer types in women, however, lung, prostate, colorectal, stomach and liver cancers are the most common cancers in males (World Health Organization. Fact sheet, 2011). Cancer can be cured by multiple approaches depending on the type and the stage of cancer that the patients have. Surgery, chemotherapy, radiation therapy, immunotherapy and hormone therapy are the main types of cancer treatments (American Cancer Society, 2016). To overcome the treatment limitations, efforts have focused on finding novel chemotherapeutic agents and fully grasping the mechanisms of its functionality. This chapter of the study investigates the anti-cancer activity of a group of imidazo[1,2-a]pyridines based (Pico) compounds against cervical cancer cell line.

4.1 Cytotoxic effects of imidazo[1,2-a]pyridines based (Pico) in cervical cancer cell line:

A panel of compounds belong to imidazo[1,2-a]pyridines (Picos) was synthesized by Prof. Adel M. Awadallah and his colleagues which includes these two compounds Pico4 and Pico6 were selected for solubility issues. Cytotoxic effects of these compounds were tested on HeLa cells using a range (0 to 50 µM) of the compounds dissolved in DMSO at 100°C for 48 hours. Cell cytotoxicity was determined by MTT assay.

4.1.1 Pico4 has a cytotoxic effect on human cervical cancer cell line

To test the cytotoxic effect of Pico4, HeLa cells were treated with arrange of 5.0µM to 50.0µM of Pico4 for 48 hours and cytotoxicity was determined by the MTT assay. Results proved that Pico4 has a strong cytotoxic impact with IC50 (37.8µM) on cervical cancer cells (Figure 4.1). Importantly, a dose dependent cytotoxic effect was observed with the concentrations (5.0 to 35.0) µM of Pico4 and little effects were noticed for concentrations higher than 35.0 µM. Furthermore, morphological signs of cell death such as floating and shrunken cells
appeared after 48 hr of the treatment (Figure 4.1c, e, g). These results show that Pico4 displays potent cytotoxicity against HeLa cells.

Figure (4.1): The cytotoxicity of Pico4 on cervical cancer cell line. HeLa cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 μM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate (a). Morphology of HeLa cells (at magnification 10X, 20X and 40X) treated either with vehicle (b, d, f) or Pico4 (c, e, g) for 48 hours. The white arrow refers to healthy cell and the black ones refer to stressed, fragmented and dead cells.
4.1.2 Pico6 has a cytotoxic effect on human cervical cancer cell line

To test the cytotoxic effect of Pico6, HeLa cells were treated with a range of 5.0µM to 50.0µM of Pico6 for 48 hours and cytotoxicity was determined by the MTT assay. Results show that Pico6 has a cytotoxic impact with IC₅₀ (42µM) on those cervical cancer cells (Figure 4.2). Importantly, a dose-dependent cytotoxic effect was observed with the concentrations (5.0 to 25.0) µM of Pico6 and little effects were noticed for concentrations higher than 35.0 µM. Furthermore, morphological signs of apoptosis such as cell shrinkage and apoptotic membrane appeared after 48 hr of the treatment (Figure 4.2c, e, g). These results showed that Pico6 displays a potent cytotoxicity against HeLa cells.
Figure (4.2): Cytotoxic effect of Pico6 on cervical cancer cell line. HeLa cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 μM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate (a). Morphology of HeLa cells (at magnification 10X, 20X and 40X) treated either with vehicle (b, d, f) or Pico6 (c, e, g) for 48 hours. The white arrow refers to healthy cell and the black ones refer to stressed cells, fragmented cells and dead cells.
All together we show her that both compounds pico4 and pico6 displayed cytotoxic effects against the tested cell line.

Therefore in the next parts of this chapter we tested the effect of imidazo[1,2-a]pyridines based (Pico) compounds on Hela cervical cancer cell line and investigated the molecular mechanism of their action.

4.2 Pico4 and Pico6 are able to induce cell death in cervical cancer cell line

Trypan blue dye exclusion assay was used to measure the impact of Pico4 on the cell viability of HeLa cells. An initial cell density of 600,000 HeLa cells/well were seeded in 6-well plates, which were developed in RPMI culture media. A concentration of 40µM for both compounds was used to treat Hela cells for 48 hours as indicated in (Figure 4.3). Viability of the cells was determined after 2 days of treatment using this assay and results were analyzed by Microsoft office Excel 2013.

Results showed that the both compounds have similar effects on the viability of HeLa cells. For example, Pico4 kills 22.5% of HeLa cells at 24 hours and 19.7% at 48 hours. Pico6 also kills 20% of HeLa cells at 24hours and about 16.5% at 48 hours as shown in (Figure 4.3a).

Taken together these data show that both Pico compounds approximately have the same action on the viability of HeLa cells (about 20%) at both time points 24 and 48 hours. Despite the fact that trypan blue assay fundamentally measures cell death rate by cell counting can be also evaluated by this assay. In support of MTT data, trypan blue results show that both compounds displayed different levels of anti-proliferation effects on HeLa cells by killing cancer cells and decreasing its number (Figure 4.3b). The proliferation rate of HeLa cells was found to be undoubtedly, inversely related to the increase in the time of treatments.

Finally we show here that both compounds induce cell death and anti-proliferative effect in the tested cell line after 24 and 48 hours of the treatment (Table 4.1 and 4.2).
Table (4.1): Percentage of cell death induced by Pico-compounds in HeLa cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of cell death after 24hr</th>
<th>% of cell death after 48hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pico4</td>
<td>22.5%</td>
<td>19.7%</td>
</tr>
<tr>
<td>Pico6</td>
<td>20%</td>
<td>16.5%</td>
</tr>
</tbody>
</table>

Table (4.2): Anti-proliferative effect of Pico-compounds in HeLa cells (percent of control).

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of cell proliferation rate after 24hr</th>
<th>% of cell proliferation rate after 48hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pico4</td>
<td>40.5%</td>
<td>25.7%</td>
</tr>
<tr>
<td>Pico6</td>
<td>10.8%</td>
<td>42%</td>
</tr>
</tbody>
</table>
Figure (4.3): Pico-compounds inhibit cancer cell viability and proliferation. Effects of Pico4 and Pico6 on cell viability of cervical cancer cells (a). HeLa cells were plated in 6-well plates and after 24 hours, cells were treated with 40 μM of Pico-compounds or vehicle. Cell viability was assessed by the trypan blue assay after 24, 48 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate. Second graph of the figure show the anti-proliferation effect of Pico-compounds on HeLa cancer cells (b).
4.3 Imidazo[1,2-a]pyridines based (Pico 4 and 6) compounds inhibit cell migration in human cervical cancer cell line

To further explore the anti-tumour activity of Pico-compounds, a scratch motility assay was performed and a significant reduction in cell migration was observed for HeLa cell line exposed to 20 μM of both Pico-compounds after 24, 48 and 72 hours of the treatment. Importantly, the anti-migration effect was more obvious at 48 and 72 hours of the treatment. While Pico4 inhibited the migratory ability of HeLa cells around 38% at 48hr and 31% at 72hr (Figure 4.4), Pico6 inhibited around 35% at 48hr and 40% at 72hr (Figure 4.5).

Figure (4.4): Pico4 inhibits migration ability of cervical cancer cells. Pico4 inhibits the migration ability of cervical cancer cells in an in vitro scratch assay. Cells were grown to 90-100% confluence and a linear wound created through the cell monolayer. Cell motility was assayed at the indicated times after addition of either vehicle (control) or Pico4 (20μM) for 72 hours. At specified time points (x-axis) cells were photographed using (4x; Olympus 1X71) and the migrated area was measured and expressed relative to zero time (y-axis). Assays were done in duplicate and two independent experiments were performed.
Figure (4.5): Pico6 inhibits migration ability of cervical cancer cells. Pico6 inhibits the migration ability of cervical cancer cells in an in vitro scratch assay. Cells were grown to 90-100% confluence and a linear wound created through the cell monolayer. Cell motility was assayed at the indicated times after addition of either vehicle (control) or Pico6 (20μM) for 72 hours. At specified time points (x-axis) cells were photographed using (4x; Olympus 1X71) and the migrated area was measured and expressed relative to zero time (y-axis). Assays were done in duplicate and two independent experiments were performed.
4.4 Imidazo[1,2-a]pyridines based (Pico 4 and 6) compounds inhibit colony formation ability of cervical cancer cell line

Colony formation ability is one important feature of cancer cells. Cell survival rate of HeLa cells treated with Pico4 and Pico6 was measured by clonogenic survival assay. Cells were treated with 40μM of Pico-compounds for 24 hours, re-plated at low density (100,300,500 and 1000) and incubated for 14 days. Results show that while HeLa cells which treated with vehicle survived at all cell concentrations, Pico4 significantly decreased HeLa survival rate (Figure 4.6). Pico4 reduced HeLa survival rate more than 80% at 1000 cell concentration. However, this was not the case for Pico6 where it has anti-survival effect on HeLa cells only at low cell concentration.

Taken together these data demonstrate that at all cell concentrations Pico4 has potent anti-survival effect on HeLa cervical cancer cells.
Figure (4.6): Pico-compounds inhibit cell survival rate of cervical cancer cells. HeLa cells were treated with 40μM Pico-compounds for 24 hours, re-plated at low densities (100, 300, 500 and 1000) and incubated for 14 days. Results show the mean percentage ± SEM of untreated cells and represent the pooled results of at least two experiments performed.
4.5 Imidazo[1,2-a]pyridines based (Pico) compounds induces Apoptosis

Pico-compounds have been shown to exert its cytotoxicity by inducing apoptosis. To investigate the mechanism by which Pico compounds exert its cytotoxic effect, HeLa cells were plated and treated with Pico compounds (40 µM) for 24 h and 48 h. Protein extracts were tested by western blotting to estimate the level of p53 and cleaved PARP which are important proteins in apoptosis (Saralamma et al., 2018). The results show that after 48 hours of Pico treatment, the cleaved PARP appears which confirms the apoptotic effect of Pico compounds. Pico-compounds also induced high level of p53.

Figure (4.7): Pico compounds induces apoptosis. HeLa cells were treated with 40 µM of Pico4 and Pico6 for 24 hours and 48h and protein extracts were analyzed by SDS-PAGE (8 %) and western blotting using antibodies to cleaved PARP and p38 and p53.
Chapter 5
Discussion
Chapter 5

Discussion

5.1 Overview

Cancer is one of the most fatal diseases in the world, for one out of three people are prone to develop cancer during their lifetime. Most of the current treatments are not sufficient to provide full cure of cancer (Chakraborty & Rahman, 2012). Regardless of major advancement in medical sciences in treating various diseases, treatment of cancer still a challenge. One approach of treating the present cancer burden is the development of targeted drugs as anticancer compounds. Those targeted treatments are engineered to target a specified features of cancer cells which makes these drugs safer to normal cells. Former studies showed that imidazo[1,2-a]pyridine has been an important biologically active moiety and many imidazo[1,2-a]pyridine based analogues have been used as lead molecules and are now under human clinical trials (Goel, Luxami, & Paul, 2016).

This study has tested of new imidazo[1,2-a]pyridine compounds for possible cytotoxic activity and reproductive inhibitory effect against cervical cancer cell line. Results showed that these compounds may be effective drugs in the treatment of cervical cancer. Furthermore, this study demonstrated that imidazo[1,2-a]pyridine compounds namely Pico4 and Pico6 inhibits cell survival and migration capacity of cervical cancer cells in vitro.

This study provides several lines of evidence that the Pico4 and Pico6 compounds, hold a promise as a novel drug to treat cervical cancer.

5.2 The Cytotoxic effects of imidazo[1,2-a]pyridines based (Pico) compounds on HeLa cervical cancer cell line

In this study, the antigrowth effect of the newly synthesized Pico4 and Pico6 was investigated against cervical HeLa cancer cell line using the MTT assay. The anti-proliferative activity was assessed after the treatment with different concentrations 5.0µM to 50.0µM of the compounds for 24 and 48 hours. It was found that this Pico4 and Pico6 have IC50s of 37.8 µM and 42 µM respectively. These results are compare with other results obtained for similar compounds. For example, 6-Substituted imidazo[1,2-a]pyridines found to exhibit excellent
activity against two cell line types of colon cancer cells namely HT-29 and Caco-2. These compounds have wide range of IC50s (6-21) against HT-29 cell line and (6-20) against Caco-2 cell line (Dahan-Farkas et al., 2011). More recent study showed a potent anticancer activity of a novel imidazopyridine compound against non-small cell lung cancer cells (Lee et al., 2013). Importantly, this compound was more potent than ours with an IC50s (1, 10 and 0.95 µM) in A549, H1299 and NCI-H596 cells, respectively. The variation in the IC50s for the same compound against different cell lines of the same type of cancer might be attributed to the different content of receptors and enzymes for each cell line (Lee et al., 2013). Another recent study of novel selenylated imidazo[1,2-a]pyridines compounds namely IP-Se-05 and IP-Se-06 showed high activity against MCF-7 breast cancer cell line with IC50 of 26.0 µM and 12.5 µM, respectively. Imidazo[1,2-a]pyridine IP-Se-06 appears to have a better antitumor effect with higher cytotoxicity at a lower concentration (Almeida et al., 2018).

Taken together, Pico4 and Pico6 compounds have a reasonable anti-proliferative effect in time dependent and dose dependent manner.

5.3 The Anti-migration effects of Imidazo[1,2-a]pyridines based (Picos) compounds on HeLa cervical cancer cell line.

In this study, a significant reduction (more than 35%) in cell migration was observed for HeLa cell line exposed to 20 µM of each Pico-compounds Pico4 and Pico6 after 48 and 72 hours of treatment. The anti-migration effect was more obvious at 48 and 72 hours of the treatment. These findings support previous data which showed that Imidazo[1,2-a]pyridine has anti-cancer effect against cervical cancer cells. Our results are similar to a previous study in which four novel imidazopyridine derivatives HIMP, M-MeI, OMP, and EtOP were synthesized and tested on different human prostate cancer cells (Ingersoll et al., 2015). These compounds were found to have different levels of suppression on cancer cell migration. Two of these compounds (M-MeI and EtOP) were able to significantly reduce prostate cancer cells migration by about 30 percent after 24 hours of 10 µM treatment. Importantly, these compounds were found to inhibit the phosphorylation of AKT kinase. Activation of AKT and its pathway is crucial for cell survival, proliferation and migration (Jung, Zheng, Jeong, Choi et al., 2013).
5.4 Colony formation ability OF Imidazo[1,2-a]pyridines.

The clonogenic assay has been established for determining the anti-survival effects of anti-cancer therapeutics on colony forming ability, in different cell lines (Rafehi et al., 2011). In this study, Cell survival rate of HeLa cells treated with Pico4 and Pico6 was measured by clonogenic survival assay. Results showed that Pico4 significantly decreased HeLa survival rate. Pico4 reduced HeLa survival rate more than 80% at 1000 cell concentration. However, this was not the case for Pico6 where it has anti-survival effect on HeLa cells only at low cell concentration. These data showed that Pico4 has potent anti-survival effect on HeLa cervical cancer cells. More recent study of 4-(3-(Tert-butylamino)imidazo[1,2-a]pyridin-2-yl)- benzoic acid compounds against kidney cancer cell line (HEK 293). Results showed that 4-(3-(Tert-butylamino)imidazo[1,2-a]pyridin-2-yl)- benzoic acid compounds reduced HEK 293 survival rate more than 90% compared to untreated control cells (Baviskar, Madaan, Preet, Mohapatra et al., 2011). Another recent study of novel selenylated two compounds IP-Se-05 and IP-Se-05 of imidazo[1,2-a]pyridines against MCF-7 cells breast cancer cell line. Both compounds IP-Se-05 and IP-Se-05 showed a high ability to inhibit MCF-7 proliferation. IP-Se-06, which inhibited cell growth around 90% compared to untreated control cells (Almeida et al., 2018).

Taken together, data in previous studies are similar to the result we have about Imidazo[1,2-a]pyridines (Pico) compounds. Showed that Imidazo[1,2-a]pyridines inhibit colony formation ability and cell growth against cancer cells.

5.5 Mechanism of action of Imidazo[1,2-a]pyridines based (Pico) compounds on HeLa cervical cancer cell line.

In cancer research field, researchers focused on inducing various forms of programmed cell death (PCD) mechanisms without affecting the vitality or integrity of normal cells (Ovadje et al., 2014). Cancer cells have been programmed to escape from apoptosis to maintain continuity and survival, so current treatments for cancer aim to activate either the intrinsic or extrinsic apoptotic pathways (Elmore, 2007; Fadeel & Orrenius, 2005).

In this present study, Imidazo[1,2-a]pyridines based (Pico) compound have been shown to exert its cytotoxicity by inducing apoptosis as evident by the high level of PARP and p53. In
recent study, 6-Substituted imidazo[1,2-a]pyridines found to exhibit excellent activity against two cell line types of colon cancer cells namely HT-29 and Caco-2. These compounds induced apoptosis in both tested cell lines. The induction of apoptosis associated with increase in cytochrome c levels within the cytosolic fraction of the cells and the activation of caspase 3 (Dahan-Farkas et al., 2011). Another group of researchers synthesized two imidazo[1,2-a]pyridines compounds and investigated apoptosis properties of both compounds against MCF-7 breast cancer cell line and HepG2 liver cancer cell line. In both cell lines MCF-7 and HepG2, the compounds induced apoptosis, increasing levels of p53 and G2/M cell cycle arrest (Androutsopoulos & Spandidos, 2018).

The apoptotic properties of a novel selective imidazopyridine cyclin-dependent kinase (cdk) inhibitor compound named AZ703 investigated against U2OS, NCI-H1299, and A549 cell lines (Cai, Byth, & Shapiro, 2006). Result showed that this compound specifically targeted cdk1 and cdk2 causing arrest in different cell cycle phases, however at higher concentration S-G2 phase arrest was obvious. The inhibition of S and G2 cdks leads to E2F-1 stabilization and expression. These results have been proved after the treatment of U2OS and NCI-H1299 cells engineered that have dominant-negative mutant E2F-1 and the data displayed that apoptosis in these cells decreased. According to these data, apoptosis induced by AZ703 compound is an E2F-1 dependent (Cai et al., 2006).

A series of novel imidazopyridine derivatives compounds (HIMP, M-MeI, OMP, and EtOP) were examined on LNCaP C-81 cells, MDA PCa2b-AI cells and the human castration-resistant PCa cells. The results displayed that HIMP and M-MeI compounds were the most potent in dose and time dependent manner (Ingersoll et al., 2015). Both of them inhibited PI3K/Akt signaling pathway, however M-MeI also suppressed androgen receptor (AR) pathways and induced cell cycle arrest in G2-phase. HIMP induced apoptosis by increasing pro-apoptotic p53 and Bax proteins levels. M-MeI decreased levels of AR targeted proteins, the prostate-specific antigen (PSA) and p66Shc. Additionally M-MeI down regulated proteins which involved in cell proliferation cyclin D1 and PCNA. Decreasing Akt protein and increasing pro-apoptotic proteins p53 and Bax were observed in M-MeI treatment (Ingersoll et al., 2015).
Figure (5.1): Imidazo[1,2-a]pyridines induce apoptotic cell death
Chapter 6
Conclusions and Recommendations
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6.1 Conclusions
This research characterized the anti-tumor activity of two novel imidazo[1,2-a]pyridines (Pico) compounds. The presented research is the first study to present the mechanism of action of these compounds where they exhibited a potent cytotoxicity against Hela cancer cells by inducing apoptosis. Accordingly, we expect from Pico4 and Pico6 to be able to reduce the growth of tumor cells in vivo.

6.2 Recommendations
1. In this study, we used one type of cancer to investigate the behavior of imidazo[1,2-a]pyridines (Pico) compounds. We here recommend to study these compounds on other types of cancers.
2. Chemical modifications are needed to improve the activity of imidazo[1,2-a]pyridines (Pico) compounds and its solubility.
3. The possible effect of these compounds on normal cells might be also important.
4. Investigating the nature of effect imidazo[1,2-a]pyridines (Pico) compounds effect would exert in vivo is recommended.
References
References


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