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Screening of synthetic chemical agents "chromenes" with potent anti-breast cancer activity

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SCREENING OF SYNTHETIC CHEMICAL AGENTS "CHROMENES" WITH POTENT ANTI-BREAST CANCER ACTIVITY

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Rabah Iratni.

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Declaration of Original Work

I, Aysha Hamad Al Meqbali, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Screening of Synthetic Chemical Agents "Chromenes" with Potent Anti-Cancer Activity"*, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision Dr. Rabah Iratni, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Cancer is the second leading cause of death worldwide. Conventional therapies cause serious side effects and, at best, merely extend the patient's lifespan by a few years. Cancer control may therefore benefit from the potential that resides in alternative therapies. There is thus an increasing demand to utilize alternative concepts or approaches to the prevention of cancer. The principal aim of this research is to screen and identify new synthetic compounds "chromenes", with high efficiency, for breast cancer therapy. In this study, we have screened four newly designed and synthesized chromenes (C1, C15, C28 and C29) for their potential anticancer activities against the highly aggressive and invasive triple negative breast cancer (TNBC) cells. We found that three (C1, C15 and C28) of the four tested chromenes exhibited a strong anti-proliferative activity against the MDA-MB-231 cells. Moreover, we found that chromenes exerts their anti-breast cancer effect through induction (i) of cell cycle arrest at the M phase confirmed by an increased expression of the M phase specific marker, p(ser10)H3 and (ii) activation of the apoptotic cell death program, revealed by increased levels of cleaved PARP , a marker of apoptosis. Further investigations are underway to elucidate the molecular mechanism(s) through which chrome exerts their effects. In conclusion, our current study provide preliminary evidences that chromenes could be a potential therapeutic compounds against the TNBC.

Keywords: Cancer, Chromenes, anti-cancer agent, apoptosis, cell viability, and cell cycle.

Title and Abstract (in Arabic)

فرز مواد كيميائيه مصنعه من (كرومين) ذات فعاليه مضاده للسرطان

الملخص

السرطان هو السبب الرئيسي ويصنف في المستوي الثاني لسبب الوفيات في العالم. العلاجات التقليدية تتسبب في أثار جانبيه خطيره, وفي احسن الظروف فهي تعمل علي مجرد اطاله عمر المريض الي بضع سنوات. ولمكافحه السرطان علينا الاستفادة من الامكانات التي تتواجد في العلاجات البديلة. وعليه هناك طلبا متزايدا علي استخدام العلاجات البديلة و المسالمة في الوقاية من السرطان. الهدف الرئيسي من هذه الدراسة هو فحص وتحديد المركبات الصناعية الجديدة "chromenes" بكفاءة عالية و منخفضه السمية لعلاج السرطان .

لعلاج سرطان الثدي في هذه الدراسة, قمنا بترتيب أربعة مركبات ل"chromenes" وتوليفها (C1، C15، C28 وC29) و التي صممت حديثا للأنشطة المضادة للسرطان المحتملة و خصيصا ضد سرطان الثدي السلبي (TNBC) الذي يعرف بالخلايا الثلاثية الشديدة العدوانية والغازية.

في دراستنا وجدنا ان ثلاث مركبات من بين الأربعة المختارة ل"chromenes" ذات مفعول قوي ومؤثر ضد خلايا سرطان الثدي. وعلاوة على ذلك، وجدنا أن تأثير "chromenes" يقوم على محاربة مرض سرطان الثدي من خلال (أ)اعتقال ووقف دورة الخلية في المرحلة M وهذا ما اكده زيادة علامه معينه للمرحلة M, P(ser10) H3) و (ب) تفعيل برنامج موت الخلية و التي كشفت عنها زيادة مستويات PARP المشقوق و الذي يرمز الى موت الخلايا المبرمج. هنالك المزيد من الدراسات و التحقيقات الجارية لتوضيح الآلية الجزيئية لمركب "chromenes". في الختام، الدراسة الحالية التي لدينا توفر الأدلة الأولية ل "chromenes" و الذي يمكن أن يكون المركب العلاجي المحتمل ضد "TNBC".

مفاهيم البحث الرئيسيه: السرطان, كرومينس, مواد ضد السرطان, موت الخلايا المبرمج, حيويه الخلايا, دوره الحياه الخلويه.

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Last but not the least, I extend my gratitude to those who taught me through the years of my master studies and bachelor studies in UAEU and to the office of graduate studies in the faculty of science and the examiner committee.

Dedication

Dedicated to my unique parents, and my sisters, brothers and all my family who were very supportive and gave me all what I need from time and nice wards to continue. I pray that Allah grants them good and blessings.

Moreover, I dedicate this thesis to my supervisors Dr. Rabah Iratni, who has really been there through the hard times.

Table of Contents

List of Figures

List of Abbreviations

DNA	Deoxyribonucleic acid
TNBC	Triple Negative Breast Cancer
Bcl-2	B-cell lymphoma-2
Bfl-1	Bcl-2 related protein
MDA-MB-231	M.D. Anderson-metastatic breast (human breast cancer cell line)
DMSO	Di-methyl sulfoxide
RIPA	Radio-Immuno-Precipitation Assay buffer
PBS	Phosphate buffer saline
DISC	Death-inducing signaling complex
Apaf-1	Apoptosis protease activating factor 1
IAP	Inhibitor of apoptosis protein
PARP	Poly(ADP-Ribose) Polymerase
H3-p ser10	Histone H3 ser 10 phosphorylation mitosis
γ-H2AX	Gamma-Histone H2A family member X
p21	Cyclin-dependent Kinase inhibiter
APC/C	Anaphase-Promoting Complex
SCF	Skp, Cullin, F-box containing complex
CKI	Cyclin-Dependent Kinase Inhibitor
CDK	Cyclin-Dependent Kinase
Cdc25	Cell division cycle 25
CIP/KIP	CDK interacting protein/Kinase inhibitory protein
INK4	Inhibitor of Kinase 4
TGF-B	Transforming growth factor beta
UAE	United Arab Emirates

Chapter 1: Introduction

1.0 Literature review

1.1 Cancer

Cancer is defined as an abnormal growth of the cells. These cells acquire defects in differentiation and in the mechanisms that control cell cycle and/or induce apoptosis in response to various stresses or DNA damage [1]. Aberrations in gene expression patterns lie at the heart of tumorigenesis. Such defects increase the ability of cancer cells to survive, grow, and divide in their original tissue and then to metastasize, which requires survival and proliferation in other environments [2,3]. The development of cancer generally requires many steps, each governed by multiple factors, some dependent on the genetic constitution of the individuals, others dependent on his or her environment and way of life [4]. Unfortunately, different cancers have different environmental risk factors, and a population that escapes one such danger is usually exposed to another.

1.1.1 Incidence of Cancer

Cancer is a major public health problem with 8.2 million deaths from cancer in the world in 2012. (WHO, 2012). In UAE, 1202 cancer cases were registered in 2007. Moreover, many studies estimated that the world population will reach 7.5 billion in 2020 and 15 million of which will be diagnosed with cancer, while 12 million people will die from cancer [4,5]. Cancer incidence and mortality has increased dramatically in both developed and developing nations. In UAE, cancer is the third leading cause of death following cardiovascular diseases and accidents.

1.1.2 Breast Cancer

Breast cancer is the most common threats in women, and the second leading cause of death following lung cancer [6]. An estimated diagnosed new cases of breast cancer reach 1.38 million per year with 458000 deaths from breast cancer worldwide [7]. They classified the breast cancer routinely according to stage, pathology, grade and expression of estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor (Her2/neu). Approximately 15% of globally diagnosed breast cancer are of the highly aggressive and invasive triple-negative (TNBC) which is defined by the absence of these targets (ER-,PR- and Her2/neu – negative) [8] .

1.1.3 Global Burden of Breast Cancer

Women are at increasingly high risk of breast cancer, due to changing exposures to reproductive and lifestyle characteristics overtime, making breast cancer a major health problem worldwide, with incidence rates increasing in most countries in the past few decades. Breast cancer risk has historically been low in developed countries compared to what is observed in the developing countries. There are several factors with this increase and is widely attributed to the ‘‘westernization’’ of lifestyles, an ill-defined surrogate for changes in factors such as childbearing, anthropometric attributes, and lifestyle characteristics. A dramatic reductions in breast cancer mortality rates in developed countries have been noticed following the introduction of screening and substantial improvements in treatment [9].

1.1.4 Causes of Breast Cancer

In general, there are many risk factors cause the breast cancer. For example, menstrual and reproductive risk factors, radiation factors, lifestyle risk factors which include body size, physical activity, smoking, alcohol and diet. Genetics, personal, and family history of breast disease is another factor which play a role in breast cancer [9]

1.1.5 Hallmarks of Cancer

Unlike normal cells, a mutation in the tumor suppressor gene P53, makes cancer cells more capable of evading the process of programmed cell death "apoptosis". To maintain nutrient supply, tumor cells induce the formation of new blood vessels through the process called "angiogenesis". In advance stage of tumorgenesis, the processes of "migration" and "invasion" happen when cancer cells detach from their tissues and migrate to invade other tissues and cause damage. According to that, the "primary tumor" can colonize in another organ and form a "secondary tumor" in a process termed "metastasis" [10]. Understanding the molecular pathways of cell division and apoptosis is essential to investigate approaches through which cancer develops. It is also critical in the process of generation of anticancer treatment**.**

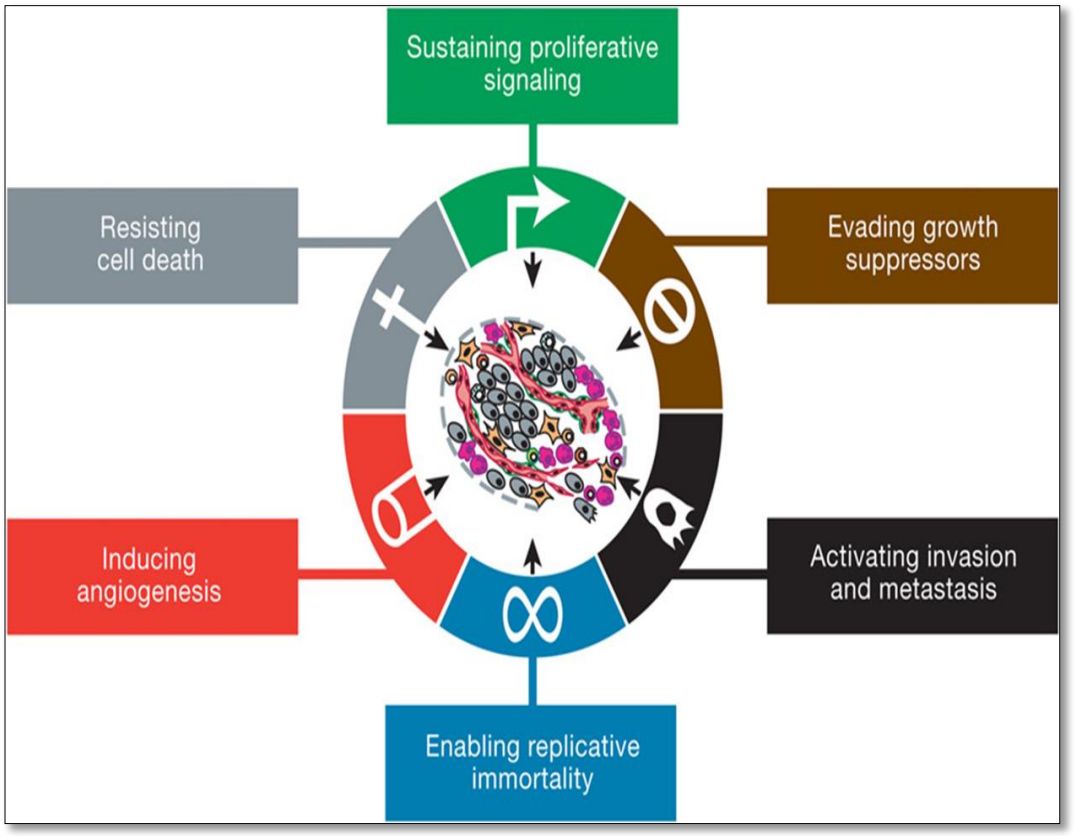


Figure 1 Hallmarks of Cancer.

1.2 Cell Cycle

Cell cycle is a process of duplication and division where cells reproduces by performing an orderly sequence of events in which it duplicates its contents and then divides in two. Cell cycle has a control system which regulate cell numbers in the tissues of the body. When the system malfunctions, excessive cell divisions can results in cancer [11].

1.2.1 The Phases of Cell Cycle

Duplication of DNA in the chromosomes and then segregation of the copies into two genetically identical daughter cells is the basic function of the cell cycle. Cell cycle

is controlled by two major phases. In S phase (DNA synthesis), chromosome duplication occurs and occupies about half of the cell-cycle time in a typical mammalian cell. After that, chromosome segregation and cell division occur in M phase (mitosis) and requires much less time. Two major events comprises in M phase, Nuclear division (mitosis) during which the copied chromosomes are distributed into a pair of daughter nuclei and cytoplasmic division (cytokinesis), which the cell itself divides in two [12, 13].

Most cell cycles have extra gap phases to allow more time for growth. G1 phase between M phase and S phase and G2 phase between S phase and M phase. G1, S, and G2 together are called "interphase" (figure 2). The two gap phases provide time for the cell to monitor the internal and external environment to ensure that conditions are suitable and preparations are complete before the cell commits itself to the major S and M phases [14].

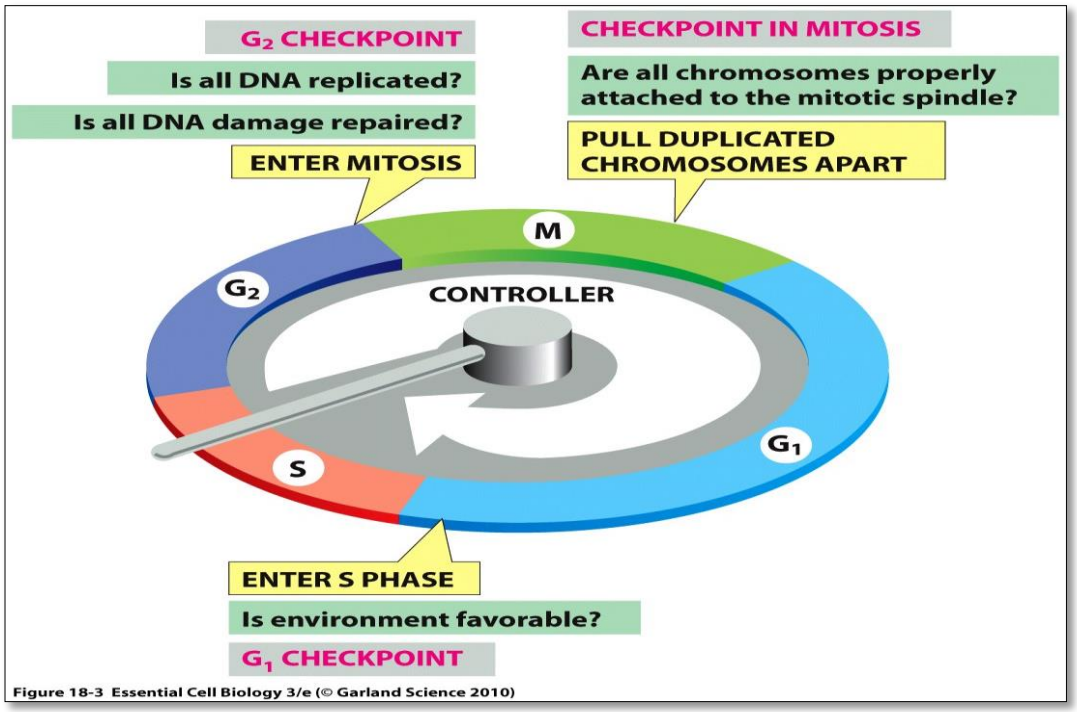


Figure 2 Normal cell cycle phases

1.2.2 Cell Cycle Control System

The cell-cycle control system is based on a connected series of biochemical switches, each of which initiates a specific cell-cycle event. In most eukaryotic cells, the cell-cycle control system triggers cell-cycle progression at three major regulatory transitions or checkpoints (figure2). The first checkpoint in late G1 where the cells enter cell cycle and proceed to S phase. The second is the G2/M checkpoint before the cell enter to M phase to make sure all DNA replicated and the environment is favorable for replication. The third is the metaphase-to-anaphase transition, where the control system stimulates sister-chromatid separation, leading to the completion of mitosis and cytokinesis. Any problems detected inside or outside the cell, will block the progression through each of these checkpoint by the control system of cyclins and cyclin-dependent kinases [10,15,16,17].

1.2.3 The role of Cyclin-Dependent Protein Kinases (CDKs)

A family of protein kinases known as cyclin-dependent kinases (CDKs) are the central components of the cell-cycle control system. Any cyclical changes in the phosphorylation of intracellular proteins that initiate or regulate the major events of the cell cycle are depend on the activities of these kinases as the cell progresses through the cycle [15] . The most important proteins that regulate these kinases are cyclins. Cyclins were originally named because they undergo a cycle of synthesis and degradation in each cell cycle. The activation of cyclin-CDK complexes that result from cyclical degradation of cyclins trigger cell-cycle events. Thus, activation of S-phase cyclin-Cdk complexes (S-CDK) initiate S phase, while activation of M-phase cyclin-Cdk complex (M-CDK) triggers mitosis. The mechanisms that control the activities of cyclin-Cdk complexes include phosphorylation of the CDK subunit, binding of CDK inhibitor proteins (CKIs), proteolysis of cyclins, and changes in the transcription of genes encoding CDK regulators. Two additional enzyme complexes play a role in the cell-cycle control system, the Anaphase promoting complex (APC/C) and SCF ubiquitin ligases, which catalyze the ubiquitylation and consequent destruction of specific regulatory proteins that control critical events in the cycle [18,19] .

1.2.4 Disrupted CDK Activating Enzyme and CDK Inhibitors

Progression of the cell cycle from one phase to another is allowed by CDKs and cyclins complexes. The activation of CDKs is controlled by the Cdc25 enzymes through dephosphorylation, which allow the transition from one phase to another. There are three members of Cdc25, the Cdc25A, Cdc25B and Cdc25C. the Cdc25A is mandatory for G1/S transition, while the Cdc25B is required for S phase and Cdc25C

mediate transition to M phase through the activation of CDK1-cyclin B. Therefore, mutation in the expression of such proteins lead to dysregulation in the cell cycle progression and can promote tumergenesis [20].

The other regulatory mechanism for CDK activity involves CDK inhibitor proteins (CKIs). CKIs are capable of exerting inhibition on cyclin-CDK complexes. There are two major families of CKI proteins, the Inhibitor Kinase 4 (INK4) family and the CDK interacting protein/Kinase inhibitory protein (CIP/KIP) family. The INK4 inhibitors consists of p15(INK4b), p16(INK4a), p18(INK4c) and p19(INK4d), while the CIP/KIP family consist of CIP1 (p21), KIP1 (p27), and KIP2 (p57),which can associate with CDK enzymes and prevent cyclin binding [15] . The activation of these inhibitory proteins is controlled by either internal or external inducers. For instance, p21 is a downstream target for the tumor suppressor transcription factor protein p53. Whereas, the activation of p15 and p27 is controlled by the Transforming Growth Factor Beta (TGF-B) proteins of the surrounding cellular environment [21]. Mutation in these inhibitors can lead to cancer. For example, mutations in CKIs such as INK4 and p21 are capable of promoting tumorgenesis and found mutated in aggressive types of cancer such as breast and lung cancers [18].

1.3 Apoptosis

Cells that are irreversibly damaged, no longer needed, or are a threat to the organism can be eliminated quickly and neatly by a process called programmed cell death or apoptosis [11].

Apoptosis depends on proteolytic enzymes called caspases, which cleave specific intracellular proteins to help kill the cell. One of these intracellular proteins is Poly (ADP-Ribose) Polymerase (PARP) which help in DNA repair and cell proliferation and serves as an apoptosis marker once it is cleaved into two large and small subunits by a caspases. Caspases present as inactive precursors called procaspases. Once activated, they cleave and activate downstream executioner procaspases, which activate other executioner procaspases, producing an amplifying, irreversible proteolytic cascade [10, 22].

There are two distinct pathways that control the activity of procaspases and leading to apoptosis. The extrinsic pathway is activated by extracellular ligands that bind to cell-surface death receptor. The death receptors recruit procaspases-8 and 10 via adaptor proteins to form the Death-inducing signaling complex (DISC). The intrinsic pathway is activated by intracellular signals generated when cells are stressed. In this pathway, cytochrome C that is released from the intermembrane space of mitochondria activates Apoptosis protease activating factor 1 (Apaf1). The Apaf1 proteins assemble into apoptosome and recruit and activate procaspase-9. Both anti-apoptotic and pro-apoptotic Bcl2 proteins regulate the intrinsic pathway by controlling the release of mitochondrial intermembrane proteins, while Inhibitor of apoptosis proteins (IAPs) inhibit activated caspases and promote their degradation [23,24,25].

The ability of cancer cells to evade programmed cell death leads to carcinogenesis[10] . This is achieved mainly through three stages of dysfunctionality in the pathway of apoptosis, which involve loss of balance between pro-apoptotic and anti-apoptotic proteins, impairment in cell death receptors and decreased caspase activity[26].

Disruption in apoptosis pathway is also linked to mutation in P53. For instance, down regulation of mutant P53 has been reported to have a major effect on reducing the formation of colonies in cancer cells and enhancing apoptosis [27].

The morphological changes in an apoptotic cells summarized in any alterations in nucleus, cell membrane and in cellular organelles. Moreover, apoptotic cells acquire a structure of rounded cells [28].

1.4 Treatment

Cancer is treated with radiation, surgery, and chemotherapy. However, these currently used clinical therapies showed limitations and high morbidity and mortality rates. Therefore, there is an urgent need for the development of new therapeutic drugs that will be more efficient, with less side effects or to be used in combination of existing ones.

The ultimate goal of all anticancer treatments is to selectively eradicate tumor cells, and the favored mechanism for this is apoptosis . A drawback of this strategy is that tumor cells frequently develop drug-resistant phenotypes by accumulating alterations in key regulators of the apoptotic pathway. The drug-resistant phenotype is often responsible for tumor regrowth and treatment failure [29].

Since microtubules play a critical role in mitosis by creating the mitotic spindle, tubulin has long been a natural target for chemotherapeutics. The major classes of microtubule-interacting agents are Taxanes and vinca and Colchicine site binders. Over expression of β-tubulin isotypes results in decreasing drug accumulation within the cancer cell due to the ability of pumping out anticancer drugs [30]. Identification

of new anti-cancer drugs that trigger cell death by acting on alternative pathways, or on different levels of a given pathway, can overcome resistance to chemotherapy. Furthermore, the availability of a broad range of anticancer drugs allows choosing between individual treatments to reduce toxicity [31,32]. Recent developments of molecular cancer therapeutics have produced new drugs that show promising antitumor effects *in vivo* [31].

1.4.1 Chromenes

Chromenes are a polycyclic organic compound that results from the fusion of a benzene ring to a heterocyclic pyran ring. These compounds play a role as a potential anticancer agent that can activate or promote apoptosis in cancer cell. Cai,S.X et al studied the effect of chromenes in T47D cells and they proved that 4H-Chromenes were identified as potent apoptosis inducers through inducing nuclear fragmentation and arrest cells at the G2/M stage followed by apoptosis in multiple human cell lines. 4H-Chromenes are developed as chemotherapeutic agents that target the colchicine binding site and serve as inhibitor for tubulin polymerization and cytotoxic against human cancer cells. For that, chromenes compound could be further developed as a potential therapeutic agent for a variety of aggressive cancers [33,34, 35] .

1.5 Objectives

The objectives of this study are to screen for new synthetic chromenes for their antiproliferative and proapoptotic effect on triple negative MDA-MB-231 breast cancer cell line and to study the mechanism(s) of action of these compounds on cancer cells.

Chapter 2: Materials and Methods

2.1 Cell Culture

Human cancer cell line of breast origin MDA-MB-231 was maintained in appropriate cell culture medium DMEM (Hyclone, Cramlington, UK) completed with 10% fetal bovine serum and 2% antibiotic (FBS) (Hyclone, Cramlington, UK). Cells was maintained in a humidified incubator at 37^{o}C in a 5% CO_2 atmosphere.

2.2 Chemical Compounds

Chromene compounds were synthesized by Dr Soleiman Hisaindee from the department of Chemistry, UAE University. These chemical compounds were suspended in DMSO at a concentration of 200 µM and stored at -80 ^{o}C .

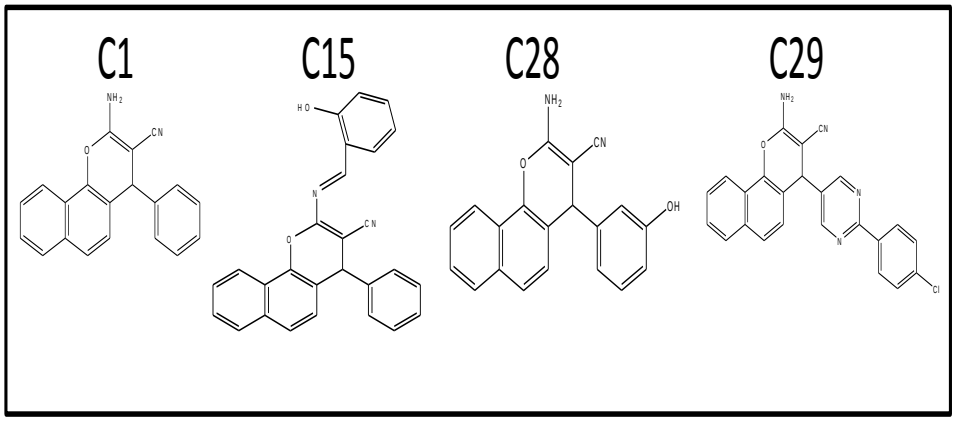


Figure 3 Chromenes compounds' structures

2.3 Cell Viability

Cancer cells were seeded in triplicate in 96-well plates at a density of 5,000 cells / well. After 24 h, cells were treated with or without various concentrations of Chromene compounds for different durations. Control cells were treated with vehicle (DMSO). The effect of drugs on cell viability was determined using Cell Cytotoxicity Kit (Abcam) according to the manufacturer's specifications. The data was presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which is assumed to be 100%.

2.4 Analysis of Morphology

MDA-MB-231 cells treated with C15 with different concentrations were examined for morphological changes using EVOS Biotechnology inverted microscope which is equipped with an indigenous camera and pictures were taken with 40X and 400X magnification.

2.5 Western Blot

Cells were seeded with 1.5 million of cells in 100mm dishes and cultured for 24h with or without the addition of C15. Cells were then washed twice with ice-cold PBS, scraped and lysed in RIPA buffer. The lysates were centrifuged at 13000 rpm for 30 min at 4ºC after incubation for 30 min in ice. Protein concentration of lysate was determined by using BCA kit. The total cell lysate were separated via SDS-PAGE and transferred onto a nitrocellulose membrane. Then, blocked for 1 h at room temperature with 5% non-fat dried milk in PBST (phosphate buffer saline with 0.05% Tween 20). Overnight incubation with specific primary antibodies was performed in at 4ºC.

Horseradish peroxidase-conjugated anti-IgG was used as secondary antibody. The membranes were then exposed to X-ray film.

2.6 Flow Cytomertic Analysis of Cell Cycle Distribution

Cancer cells were seeded with 300,000 cells in 60mm culture dishes and cultured for 24 h before addition of various concentrations of C15 compound or equal volume of vehicle (DMSO) as control. After incubation for the indicated time, cells were harvested by trypsin release, washed twice with ice-cold PBS, resuspended in PBS, fixed with an equal volume of 100% ethanol and incubated for at least 12h at -22^{0}C. Before flow cytometry analysis, cells were pelleted, washed twice with PBS. Then Stained with cell cycle kit (Millipore) and analyzed with MUSE cell analyzer. Percentage of cells in G1, S and G2/M phases was determined using the FlowJo software.

2.7 Statistical Analysis

The statistical analysis were done using SPSS version 21. Data were reported as group mean ± SEM. A student's t-test for paired or unpaired values was performed. Significance for all statistical comparisons was set at *p* value of <0.05 using a two-tailed test.

Chapter 3: Results

3.1 Effect of the Chromenes on the Cellular Viability of the MDA-MB-231 Breast Cancer Cell Line.

To examine the anticancer effect of chromenes on breast cancer cell line, we first measured the effect of various concentration of chromenes (C1, C15, C28 and C29) on the proliferation of MDA-MB-231 cell line. Our results showed a nice decrease in the cellular viability of MDA-MB-231 cell line when exposed to various concentration of Chromenes (C1, C15 and C28) but not with C29. Furthermore, treatment with 2.5 µM of chromenes 1, 15 and 28 for 24 (Figure 4A) and 48 Hrs (Figure 4B) resulted in approximately 50% decrease in the cell viability of MDA-MB-231 cell line. The exposure of MDA-MB-231 to chromenes decreased cellular viability in a concentration and time-dependent manner compared to a control cells treated with vehicle (ethanol).

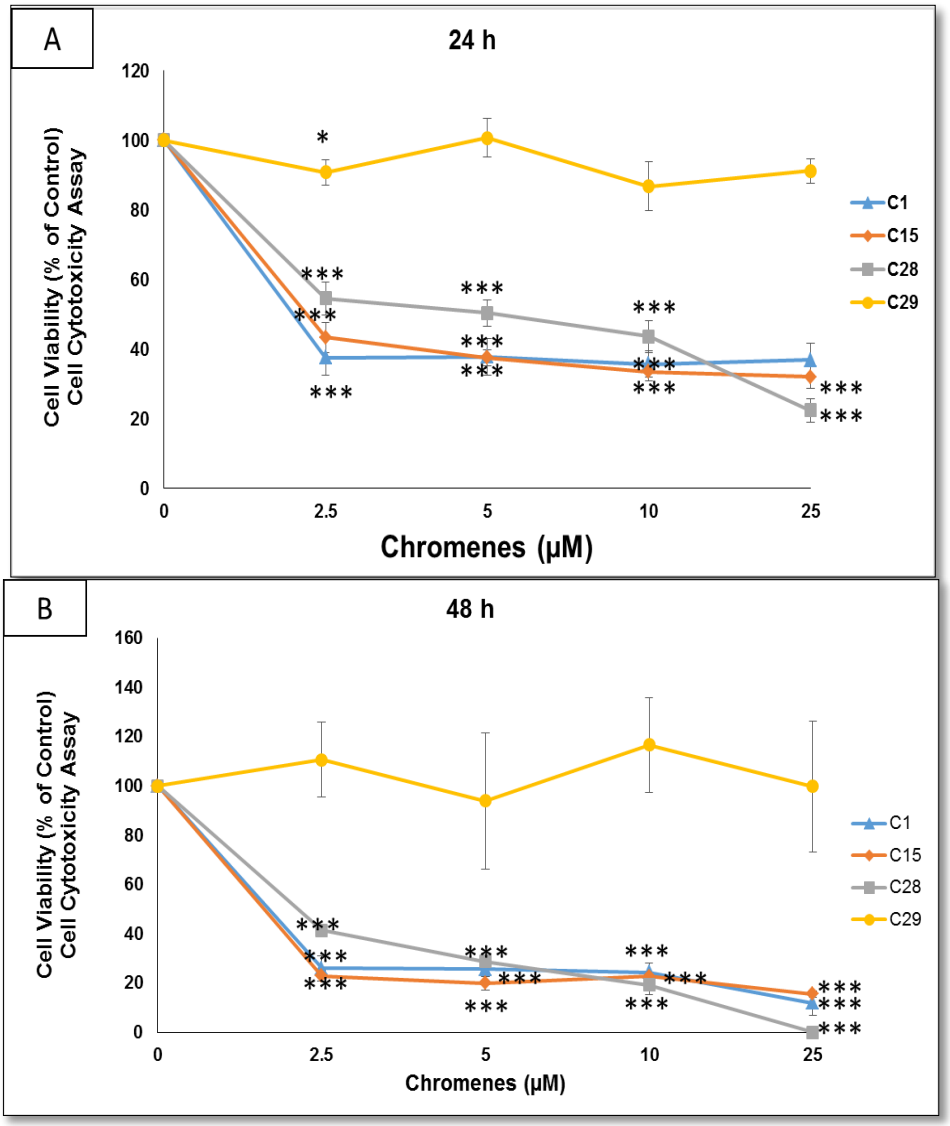


Figure 4 Inhibition of cellular viability of MDA-MB-231 cells by Chromenes in concentration and time dependent manner. MDA-MB-231 cells were treated with and without the indicated concentration of chromenes (1, 15, 28, and 29) for 24hrs (A) and 48hrs (B). viability monitored as described in materials and methods. Data represent the mean of four independent experiments carried out in triplicate. Statistical analysis for cell viability data was performed and reported as a group of ±SEM. p value of <0.05 was considered statistically significant.

3.2 Morphological Changes Induced by Chromene 15 in MDA-MB-231 Cell Line.

Next, we focused our attention to C15 only. To understand the mechanism(s) of action of this compound (C15) in inhibiting the cellular viability, we first looked at the morphology of MDA-MB-231 in control and C15-treated cells for 24 hours. As it is shown in figure 5, light microscopy observation revealed that the number of MDA-MB-231 cells in the presence of C15 significantly decreased compared to the control. Morphological observation also revealed that treated cells exhibited a rounded-shaped and smaller size a characteristic of apoptotic cells (Figure 5).

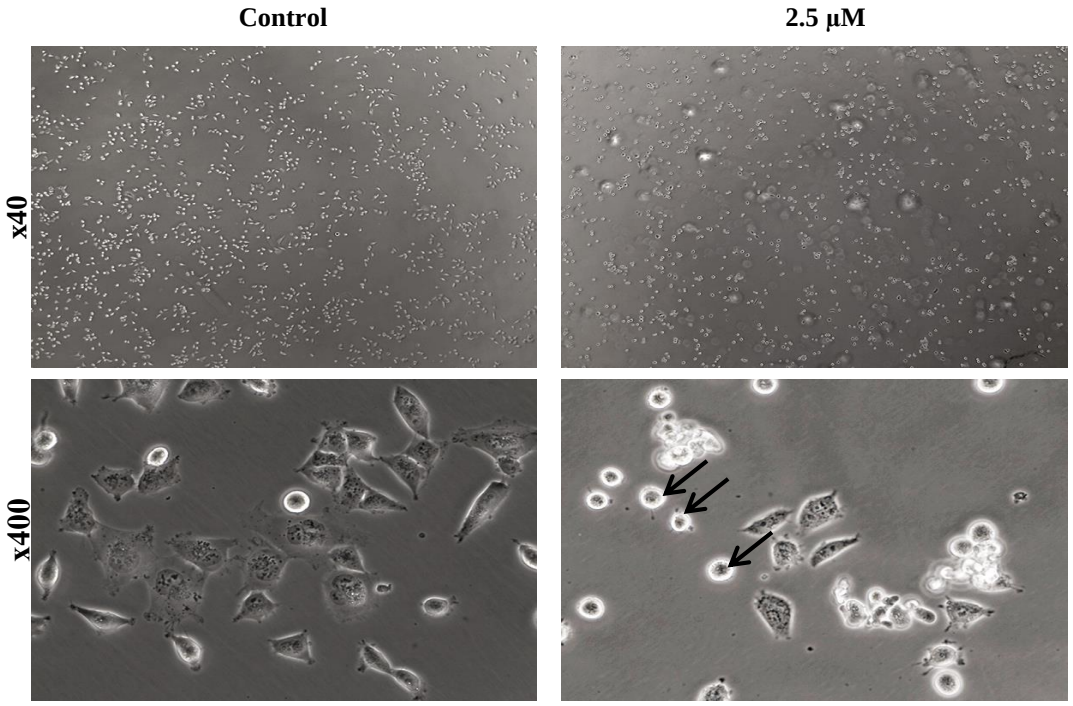


Figure 5 Morphological changes in MDA-MB-231 cells treated with C15 for 24 hrs. Images taken at 40x and 400x magnifications. Cells were examined under EVOS XL Core Cell Imaging System (Life Technologies).

3.3 Significant Induction Of Apoptosis By C15 In Mda-Mb-231 Cells.

Further investigation of the mechanism of cell death induced by C15 on MDA-MB-231 cells, we studied the expression of cleaved PARP in treated cells with different concentrations of C15 for 24hrs. As shown in figure 6, a high accumulation of cleaved PARP which is considered as an apoptosis marker was observed, and thus suggesting the occurrence of apoptosis in C15-treated MDA-MB-231 cells.

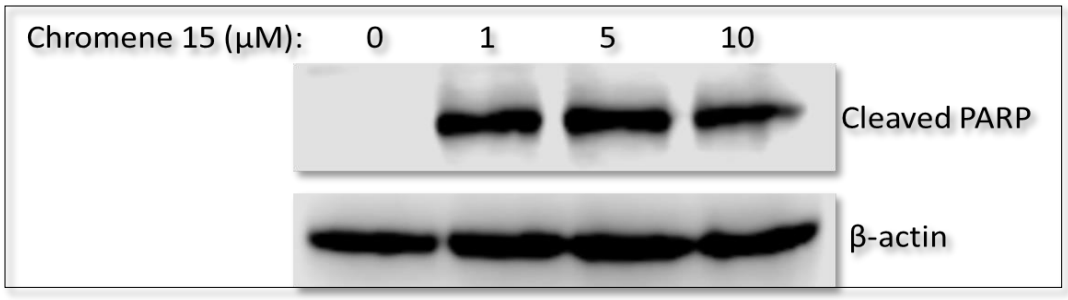
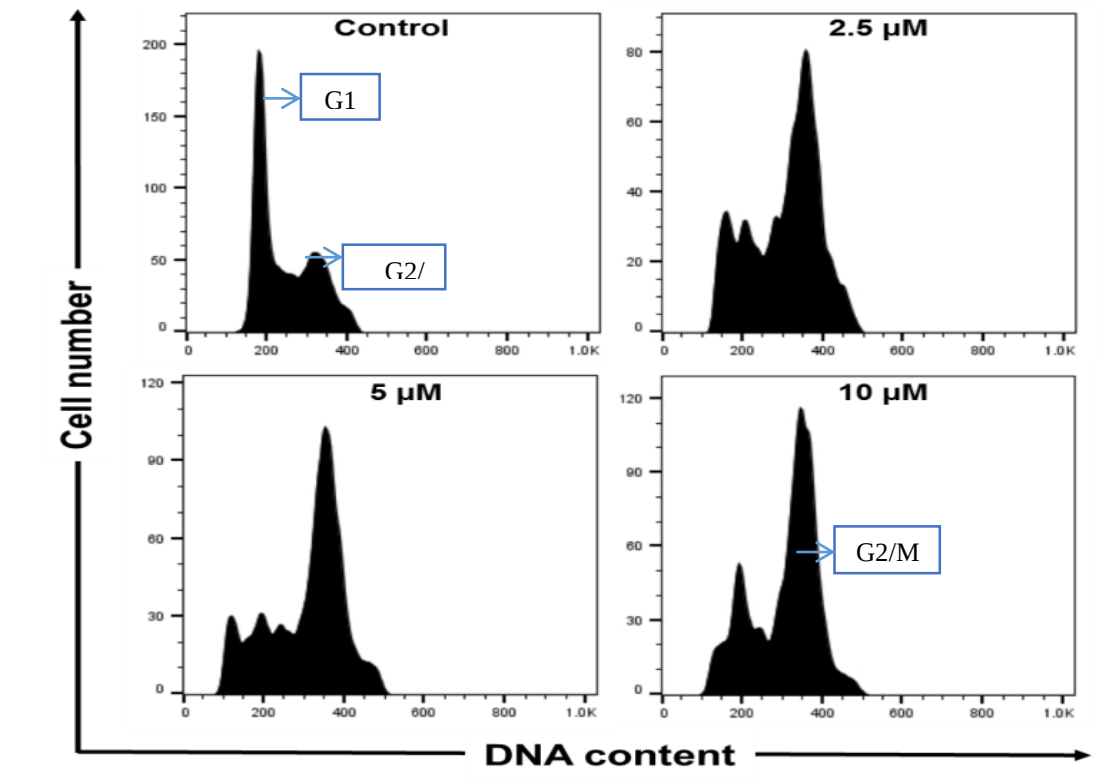


Figure 6 Induction of apoptosis by C15 in MDA-MB-231 cells. Cells were treated for 24 hrs with different concentrations of the C15. Western blot analysis was carried out using anti-cleaved PARP antibody.

3.4 C15 Leads To Mitotic Arrest on Mda-Mb-231 Cell Line

The ability of an anticancer drug to affect cell cycle distribution can provide information regarding its cytotoxic mechanism(s) of action. Therefore, we investigated the effect of C15 on cell cycle distribution. To achieve this, cells were treated with increasing concentrations of C15 for 24 hrs and then cell cycle phases were measured. At the concentration of 2.5 μM, C15 caused an obvious G2/M arrest as it is shown in figure 7. Indeed the population of G2/M increased significantly, from 30 to over 60%, as the concentration of C15 increased indicating that C15-treated MDA-MB-231 cells underwent cell cycle arrest at G2/M phase.



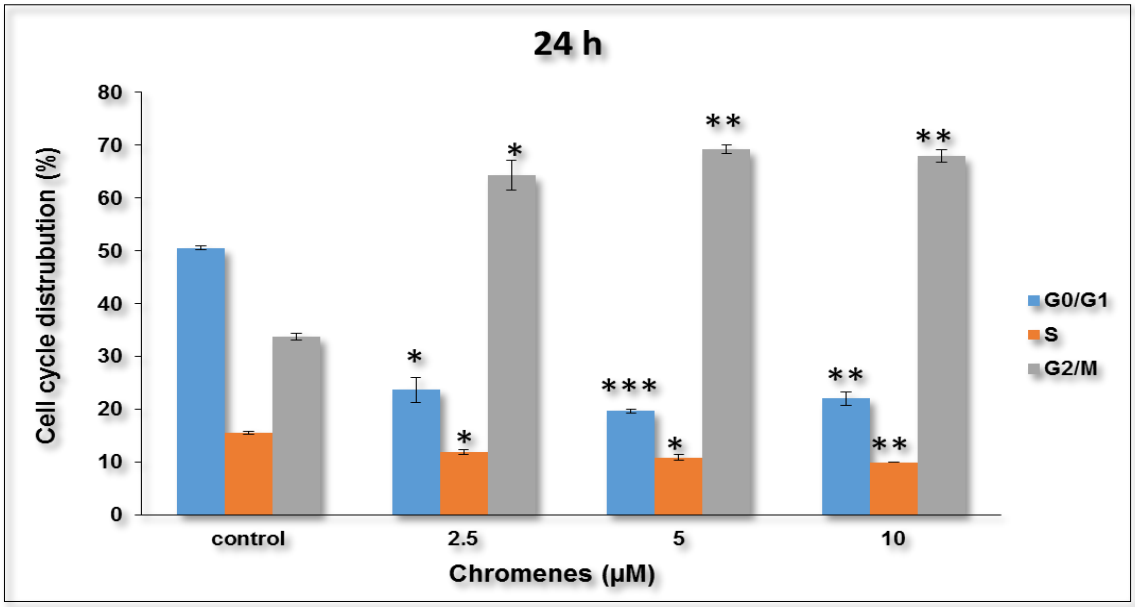


Figure 7 Induction of G2/M cell cycle arrest by C15 in MDA-MB-231 cell line. Cells were exposed to various concentrations of C15 for 24h. After treatment cell analyzed for cell cycle distribution using flow cytometry. Data are reprentative of three independent experiments. Percentage of cells in G1, S and G2/M phases was determined using the FlowJo software. *p* value of <0.05 was considered statistically significant.

3.5 C15 Leads To Upregulation Of P21 Expression In Mda-Mb-231 Cells

It is well known that the cyclin dependent kinase (CDK) inhibitor p21 cause a cell cycle arrest when cells are exposed to anticancer compounds [15]. Because we found that C15 induces cell cycle arrest at G2/M phase in C15-treated cells, we decided to examine the level of p21, by Western Blot, in response to C15. As shown in figure 8, the expression of p21 increased in MDA-MB-231 cell treated with low concentrations (1 and 5 µM) of C15. Interestingly a higher concentration of C15 (10 µM) did not show a change in p21 level. Still we cannot rule out the possibility that p21 increased at early time point but due to massive cell death at high concentration, the protein undergoes degradation. From this result we can conclude that C15 induce cell cycle arrest through induction of the CDK inhibitor, p21.

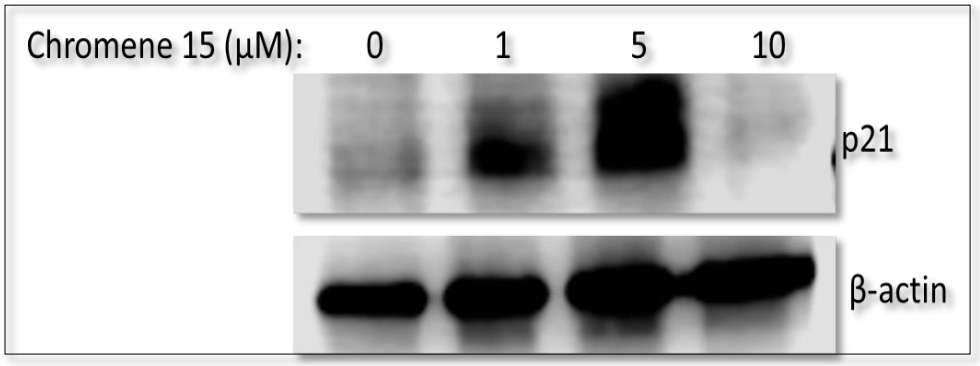


Figure 8 Expression of p21 in C15-treated MDA-MB-231 cells. Cells were treated with various concentrations of C15 or vehicle as a control for 24 h and the expression of p21 was estimated by Western Blot.

3.6 Phosphorylation Status of H3 (Ser 10) Detected In C15-Treated Mda-Mb-231 Cells

To determine whether C15 induce cell cycle arrest specifically at mitosis or G2 phase, we analyzed the expression of G2 and M phase specific markers, cyclin B and

histone H3-phospho (Ser 10), respectively by Western Blot. Figure 9 showed that the level of C15- treated cells significantly increase the phosphorylation level of H3-phospho (Ser 10) in dose-dependent manner in response to C15. The level of cyclin B1 on the other hand remained unaffected (Figure 9). Altogether, our results indicates that C15 induces a specific cell cycle arrest at the M phase.

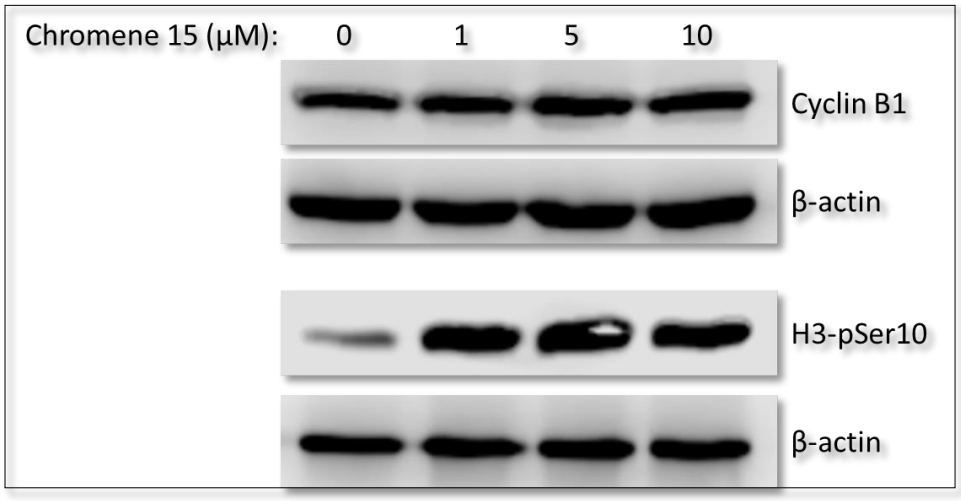


Figure 9 Expression of cell cycle regulator in C15-treated MDA-MB-231.Western blot analysis of phosphor (ser10)-H3 and cyclin B1 in MDA-MB-231 cells exposed for 24 h with ethanol or indicated concentrations of C15.

3.7 Effect Of C15 In The Dna Damage Of Mda-Mb-231 Cells

Finally, we tested whether C15 mediates its effect through induction of DNA damage as it is the case for numerous ancticancer compounds. Toward this, we examined the level of the marker of DNA damage ɣ-H2AX in treated-cells by Western Blot. As it is shown in figure 10, C15 did not induce any significant increase on the level of ɣ-H2AX, thus indicated that C15 mediated effect is independent of DNA damage.

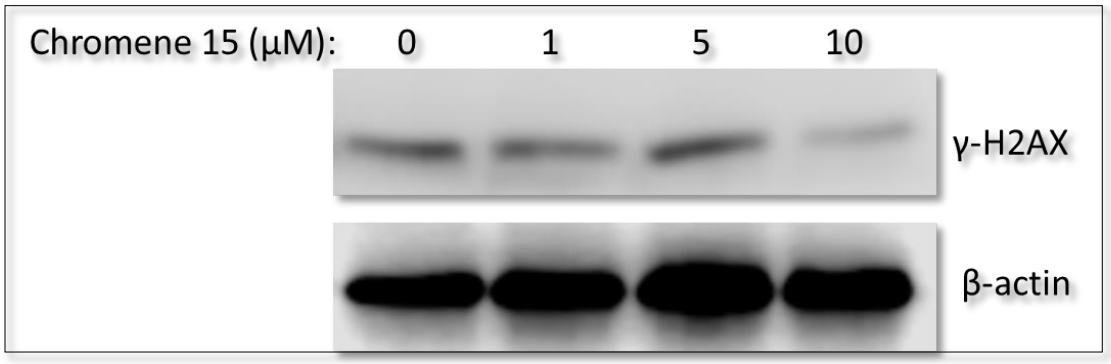


Figure 10 Effect of C15 in the DNA damage of MDA-MB-231 cells. C15 did not cause a DNA damage in MDA-MB-231 cells. western blot analysis of phospo-H2AX in MDA-MB-231 cells exposed for 24 h with indicated concentrations of C15 or vehicle as a control.

Chapter 4: Discussion

The main goal of anticancer drugs is to inhibit cell cycle progression and induce cell death. Many natural products play a role in these two events and act as anticancer drugs in the field of cancer treatment. Hence, we started our investigation for potential anticancer effect of C15 by testing its cytotoxicity effect and its ability to induce cell death in MDA-MB-231 cell line.

In our study, we have shown that chromenes play a role in inhibiting cellular viability and inducing apoptosis. This result was clear in the cytotoxicity assay and in the morphology of the MDA-MB-231 cells treated with different concentrations of chromenes. Apoptosis was further confirmed by PARP cleavage expression which was detected by Western blot.

Furthermore, cell cycle represents another target in developing chemotherapy against cancer cells [36]. Interestingly, our results showed that C15 induced cell cycle arrest at G2/M phase and more precisely at the M phase. This result was further supported by Western blotting analysis of specific marker to mitotic arrest which revealed an increased level of H3-phospho (Ser10). Interestingly, we found that cell cycle arrest correlated with an upregulation of the CDK inhibitor p21. Indeed, p21 is known to block cell cycle by binding to CDK and thus inhibiting its activity. Several studies suggested a pro-apoptotic role for p21. Indeed, it was revealed that apoptosis coexisted with induction of p21 without defining whether p21 is needed for the induction of apoptosis [37]. Hence, we cannot rule out a potential involvement of p21 in the induction of apoptosis in response to chromenes.

Since microtubules play a critical role in mitosis by creating the mitotic spindle, tubulin has long been a natural target for chemotherapeutics [38]. The most commonly prescribed successful anticancer therapies, that target microtubules are Paclitaxel and Docetaxel. These two chemotherapy agents play a role in Taxane binding site which serve as one of the major classes of microtubule-interacting agents [39,40] . The other two classes are Vinca and Colchicine site binders. Vincristine and Vinblastine are clinically applied microtubule-interacting agents that bind with Vinca alkaloid site [41] . The widespread clinical use of these agents in a variety of cancers reveal the importance of tubulin and its role in cancer. Interestingly, in our study, we showed that chromenes induces targeted breast cancer cells through induction of mitotic block with subsequent activation of the apoptotic pathway. We also showed that mitotic block is not a consequence of genotoxic effect of the chromenes as no significant DNA damage was detected in treated-cells. Hence we can speculate that chromenes might induce mitotic block through targeting the assembly of the mitotic spindle or inducing microtubule depolymerization, possibly by targeting the tubulin. Further investigation to examine this hypothesis is underway in the lab.

Chapter 5: Conclusion

In conclusion, in this study we showed that the newly designed chromenes affect significantly inhibited cellular viability of TNBC in a concentration- and time-dependent manner. Moreover, chromenes induced cell cycle arrest and apoptosis and upregulated the CDK inhibitor p21. Results from this study provide preliminary evidence that chromenes possesses a potential anti-cancer activity against the most aggressive and invasive form of breast cancer and therefore deserves more attention for further investigations to study the mechanism(s) of action of these compounds.

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