ผลของสารประกอบคูมาริน CHA-01 จากต้นส่องฟ้าดง *Clausena harmandiana* (Pierre) Guillaumin ต่อฤทธิ์ต้านการเพิ่มจำนวนในเซลล์ไลน์มะเร็งมนุษย์

นางสาวอรพรรณ ภารสุคนธ์

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางอุตสาหกรรม ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECT OF COUMARIN COMPOUND CHA-01 *Clausena harmandiana* (Pierre) Guillaumin ON ANTI-PROLIFERATION ACTIVITY IN HUMAN CANCER CELL LINES

Miss Orapan Parnsukhon

จุฬาลงกรณ์มหาวิทยาลัย
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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Industrial Microbiology
Department of Microbiology
Faculty of Science
Chulalongkorn University
Academic Year 2014

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Thesis Title	EFFECT OF COUMARIN COMPOUND CHA-01 *Clausena harmandiana* (Pierre) Guillaumin ON ANTI-PROLIFERATION ACTIVITY IN HUMAN CANCER CELL LINES
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อรพรรณ ภารสุคนธ์ : ผลของสารประกอบคูมาริน CHA-01 จากต้นส่องฟ้าดง *Clausena harmandiana* (Pierre) Guillaumin ต่อฤทธิ์ต้านการเพิ่มจำนวนในเซลล์ไลน์มะเร็งมนุษย์ (EFFECT OF COUMARIN COMPOUND CHA-01 *Clausena harmandiana* (Pierre) Guillaumin ON ANTI-PROLIFERATION ACTIVITY IN HUMAN CANCER CELL LINES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ชุลี ยมภักดี, 109 หน้า.

Clausena harmandiana (Pierre) Guillaumin หรือ ส่องฟ้าดง จัดอยู่ในวงศ์ Rutaceae ในงานวิจัยก่อนหน้า ได้แยกสารบริสุทธิ์จากส่วนใบของ *C. harmandiana* พบว่าเป็นสารประกอบคูมารินชนิดหนึ่งโดยให้ชื่อว่า CHA-01 มีฤทธิ์ยับยั้งวิถีการส่งสัญญาณของแคลเซียมในยีสต์*Saccharomyces cerevisiae* สายพันธุ์กลายที่ขาดยีน *ZDS1* (Δ*zds1*) แต่ความรู้เกี่ยวกับฤทธิ์ทางชีวภาพของสารประกอบคูมาริน CHA-01 ยังมีอยู่น้อยมาก จากที่เคยมีรายงานว่าสารประกอบคูมารินอื่นๆบางชนิด มีฤทธิ์ต้านมะเร็งได้ จึงนำมาสู่วัตถุประสงค์ของงานวิจัยนี้คือเพื่อศึกษาฤทธิ์ต้านการเพิ่มจำนวนเซลล์ของ CHA-01 ต่อเซลล์ไลน์มะเร็ง และศึกษาโมเลกุลเป้าหมายที่เกี่ยวข้องกับฤทธิ์ทางชีวภาพของ CHA-01 ผลการทดลองพบว่า CHA-01 มีฤทธิ์ต้านการเพิ่มจำนวนของ Jurkat (เซลล์ไลน์มะเร็งเม็ดเลือดขาวชนิดลิมโฟไซท์), KATO III (เซลล์ไลน์มะเร็งกระเพาะอาหาร) และ THP1(เซลล์ไลน์มะเร็งเม็ดเลือดขาวชนิดมอนอไซท์) โดยวิธี MTT assay พบว่าเซลล์ไลน์มะเร็งชนิด Jurkat มีความไวต่อ CHA-01 มากที่สุด มีค่า IC_{50} เท่ากับ 0.67±0.01 ไมโครโมลาร์ และไม่มีความเป็นพิษทั้งต่อเซลล์เม็ดเลือดขาวและเซลล์เม็ดเลือดแดงของคนปกติ นอกจากนี้ CHA-01 สามารถหยุดวัฏจักรการแบ่งเซลล์อยู่ที่ระยะ S อีกทั้งเหนี่ยวนำให้เซลล์เกิดการตายแบบอะพ็อพโตซิสและสามารถยับยั้งการสังเคราะห์ดีเอ็นเอ จากการวิเคราะห์ทางโปรติโอมิกส์ของเซลล์ไลน์ชนิด Jurkat ที่สัมผัสกับ CHA-01 นาน 60 นาที พบโปรตีนทั้งสิ้น 861 ชนิด ได้คัดเลือกโปรตีนที่มีหน้าที่ที่เกี่ยวข้องกับการเพิ่มจำนวนของเซลล์ วัฏจักรการแบ่งเซลล์ และการสังเคราะห์ดีเอ็นเอ มาศึกษาการแสดงออกของยีนที่ระดับ mRNA โดยวิธี quantitative real-time PCR พบว่ามีการลดของระดับ mRNA และระดับโปรตีนของยีน *MCM4 POLD3* และ *NPM1* ในขณะที่มีการเพิ่มของระดับ mRNA และระดับโปรตีนของยีน *PRKACB* และ *FADD* แสดงว่า CHA-01 ออกฤทธิ์กดการแสดงออกของยีน *POLD3* และ *MCM4* ทำให้มีการหยุดการสังเคราะห์ดีเอ็นเอ ส่งผลให้หยุดวัฏจักรการแบ่งเซลล์ที่ระยะ S ทั้งนี้ CHA-01 น่าจะออกฤทธิ์ไปเพิ่มการแสดงออกของยีนและเพิ่มระดับโปรตีน *PRKACB* และ *FADD* อันเป็นผลให้เซลล์เข้าสู่การตายแบบอะพ็อพโตซิส และทำให้เซลล์เหลือรอดชีวิตลดลงอย่างมากแบบมีนัยสำคัญในที่สุด

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557

5472154623 : MAJOR INDUSTRIAL MICROBIOLOGY
KEYWORDS: COUMARIN / ANTICANCER / CELL CYCLE ARREST / DNA SYNTHESIS INHIBITION / APOPTOSIS / JURKAT

ORAPAN PARNSUKHON: EFFECT OF COUMARIN COMPOUND CHA-01 *Clausena harmandiana* (Pierre) Guillaumin ON ANTI-PROLIFERATION ACTIVITY IN HUMAN CANCER CELL LINES. ADVISOR: ASST. PROF. CHULEE YOMPAKDEE, Ph.D., 109 pp.

Clausena harmandiana (Pierre) Guillaumin or Song faa dong (in Thai), is classified in Family Rutaceae. Previous study, a coumarin compound designated CHA-01 was isolated from leave extract of *C. harmandiana* with inhibitory activity against calcium signaling in a *ZDS1* null mutant yeast *Saccharomyces cerevisiae* (Δ*zds1*). However, not much has been known on biological activity of this coumarin. In the past, some other coumarins were reported to contain anti-cancer activity. The aims of this research were to evaluate anti-proliferative activity of CHA-01 on several cancer cell lines and to study on molecular target(s) of CHA-01 in the Jurkat cells. The results revealed that CHA-01 showed anti-proliferative activity in several cell lines including Jurkat (Human acute T cell leukemia cell line), KATO III (Human gastric carcinoma cell line) and THP1 (Human acute monocytic leukemia cell line) by MTT assay. Jurkat T cell line was the most sensitity cell line to CHA-01 treatment with IC_{50} value of 0.67±0.01 μM. It contained no cytotoxic activity against normal white blood cells and red blood cells. The CHA-01 treated Jurkat T cells arrested in S phase of the cell cycle. In addition, CHA-01 induced the cell death by apoptosis and inhibited DNA synthesis. Proteomic study of CHA-01 treated Jurkat cells for 60 minutes found total of 861 proteins. Among these, the proteins involved in cell proliferation, cell cycle and DNA synthesis were chosen. Some selected genes were cloned and studied for gene expression by quantitative real-time PCR. *MCM4, POLD3* and *NPM1* genes as well as their products were found to be down regulated while those of *PRKACB* and *FADD* were up regulated. The results suggested that CHA-01 might inhibit *POLD3* and *MCM4* genes expression that cause DNA synthesis inhibition, resulting in S phase cell cycle arrest. In addition, CHA-01 might activate *PRKACB* and *FADD*, hence the cells died from apoptosis and finally, the number of viable cells significantly decreased after CHA-01 treatment.

Department:	Microbiology	Student's Signature	.....
Field of Study:	Industrial Microbiology	Advisor's Signature	.....
Academic Year:	2014		

ACKNOWLEDGEMENTS

I am sincerely grateful to Asst. Prof. Chulee Yompakdee, Ph.D., my advisor and Assoc. Prof. Tanapat Palaga, Ph.D., Sittiruk Roytrakul, Ph.D. for their supporting, assistance, valuable suggestion and encouragement throughout this research work. In addition, I also express my appreciation to all thesis committee, Prof. Sirirat Rengpipat, Ph.D. and Asst. Prof. Naraporn Somboonna, Ph.D.

I would like to thank Akkaraphol Srichaisupakit, Ph.D., Ms. Anyaporn Sangkaew, Ms. Atchara Paemanee and Ms. Suthathip Kittisenachai for they constructive advice and encouragement. Furthermore, I thank Mr. Jumpol Sopanaporn and Ms. Orrarat Sangkaew for their kind help, suggestions and encouragement throughout my research. I would like to thank the member of 2015 and 2017 laboratory room for the suggestion and supporting equipments.

I would like to thank Department of Microbiology, Faculty of Science Chulalongkorn University and Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC) for providing the facilities and all instruments for my research. Also, I do wish to thank all members and staff of them for their kind assistance in this research. Furthermore, I thank THE 90TH Anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund) for supporting the subvention.

Finally, special thank for my lovely family, father, mother and my sister who encourage my education and respect my decision.

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CHAPTER I
INTRODUCTION

Cancer is one of leading causes of death in Thailand as well as in developed countries. Frequently observed types of cancer in Thailand are such as cervical cancer and breast cancer in female and liver cancer and lung cancer in male. Causes and risk factors of cancer are external factors, for example**s** carcinogens-contaminated food and drink such as aflatoxin in molded foods, hydrocarbon in grilled foods, nitrosamine in preserved foods and food coloring agents made from dye used in textile industry. Habits such as smoking and drinking behaviors, and exposure ultraviolet ray in sunlight. Internal factors, play a role with congenital genetic disorder that poses high risk of leukemia, if there is immunodeficiency or malnutrition lacking vitamin C, for example (National Cancer Institute (Thailand), 2015). Chemotherapy is the use of drug to treat cancer. Drug which kill cancer cells also possessed side effects that some of normal cells are also killed.

In present, scientists and researchers are studying about properties of herbs for therapy and the treatment of various symptoms and diseases. The research and development of natural product, traditional medicine, screening of novel bioactive compounds for development of new drugs are widespread and trending. There are problems of currently used drugs in modern medicine such as undesired side effects, drug resistance, inadequate efficacy and novel emerging diseases. In addition, drugs imported from other countries are high in cost, resulting in a balance of trade deficit of drug industry in country. So there are still needs in improvement and search for novel drugs which is one of the major interests in current health research topics.

Calcium signaling pathway is one of pathways that has important roles in controlling cellular proliferation, T-cell activation, secretion, muscle contraction and neurotransmitter release in higher eukaryotes (Clapham 1995). Substances, which inhibit calcium signaling pathway, has potential to develop into medicines. Potential drugs are vary, depend on the type of molecular targets. For examples, anti-cancer, anti-inflammatory, anti-fungal and much more to be incidental. The inhibitor of calcium signaling pathway is expected to be of great pharmaceutical interest (Shitamukai et al. 2000). Previous studies in our laboratory showed that the crude extract from leaves of *Clausena harmandiana* (Pierre) exerted a strong inhibitory activity on the Ca^{2+}-signal mediated cell-cycle regulation in the yeast *Saccharomyces cerevisiae*. Using the Δ*zds1* proliferation assay (Wangkangwan et al. 2014) on activity-guided fractionation and purification in combination with several chromatography techniques, a pure coumarin compound, CHA-01, was obtained.

C. harmandiana (Pierre) is commonly known in Thai as Song faa dong. It is a Thai medicidal herb in family Rutaceae and can be found in the East, North-East and South of Thailand. Young leaves are edible as vegetable dish and used as traditional medicines for the treatment of several illnesses such as fever, headache and stomachache. However, little is known on *C. harmandiana* (Pierre) biological activity. There has been a report that it possesses some biological activity such as antibacterial activity (Maneerat et al. 2012). Thus, it is interesting for studying to find other bioactive compound from *C. harmandiana*. From the research of Suauam (2011), whose examined the bioactive and pure compound from *C. harmandiana* by Δ*zds1* proliferation assay, found that a coumarin compound exhibited calcium

signaling pathway inhibition in Δ*zds1* yeast strain. Furthermore, the coumarin has no cytotoxicity on yeast cell. Yeast genetics studies showed that calcineurin is a molecular target of the coumarin compound (Suauam 2011). Calcineurin is a conserved protein from yeast to human (Kingsbury and Cunningham 2000). Suauam (2011) examined the effect of the coumarin compound on Jurkat T cell, and found that the coumarin compound inhibited the production of Interleukin 2 (IL-2). Molecular target of coumarin compound was previously identified as calcineurin. In T-lymphocyte, calcineurin is activated by calcium and calmodulin. Calcineurin activates nuclear factor of activated T cell (NFAT), a transcription factor, by dephosphorylating it. The activated NFAT is translocated into the nucleus, where it up-regulates the expression of cytokine genes (*e.g.* IL-2). Calcineurin is target of a class of drugs called calcineurin inhibitors such as FK506 (Tacrolimus) and Cyclosporin A. The calcineurin inhibitors are the potentially effective immunosuppressive drugs. The coumarin compound did not show acute cytotoxicity on Jurkat T cell. So the coumarin compound from *C. harmandiana* (Pierre), which can inhibit calcineurin, may be a novel immunosuppressant. Coumarins naturally present in many plants have been found to exhibit various biological activities including anticancer, anti-inflammatory, anticoagulant, antimicrobial, antioxidant and anti-allergic properties (Wu et al. 2009, Riveiro et al. 2010). It has been reported that certain coumarin compounds inhibit the cell growth in various types of cancer cell lines (Marshall et al. 1994, Chuang et al. 2007).

In this research, the anti-proliferative and acute or chronic cytotoxicity effect of coumarin compound (CHA-01) from *C. harmandiana* on various human cancer cell lines was examined. The coumarin's role on the cell cycle progression, induced

apoptosis and DNA synthesis inhibition in the affected cancer cell lines were also investigated. The study of proteins involving in anti-proliferation activity in cancer cell lines affected by the CHA-01 were investigated for molecular insights and for the development of cancer drug from Thai herbal medicine.

1.1 Research methodologies

1.1.1 To study the effect of the CHA-01 on the anti-proliferation on cancer cell lines (Jurkat T cell, THP-1 and KATO III) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Proliferation assay

1.1.2. To study on cytotoxic effect of the CHA-01 on normal white blood cells by MTT Proliferation assay

1.1.3. To study on cell cycle progression in the CHA-01 treated cancer cell lines by flow cytometry

1.1.4. To study the apoptotic effect of the CHA-01 on the cancer cell lines by flow cytometry

1.1.5. Proteomic analysis of proteins from CHA-01 treated cancer cell lines

1.2 Expected Outcomes

1.2.1. Identify biological activities of the CHA-01 from the leaves of *C. harmandiana* (Pierre)

1.2.2. Elucidate proteins associate with anti-proliferation activity of CHA-01 in human cancer cell line

CHAPTER II
LITERATURE REVIEW

2.1 Overview of cancer

Cancer is one of leading causes of death in human. World Health Organization (WHO) in 2012, reported approximately 14 million new cancer cases and 8.2 million death. The number of new cases is expected to rise by about 70% in next year. The most common causes of cancer death are lung cancer (1.59 million deaths), liver cancer (745,000 deaths), stomach cancer (723,000 deaths), colorectal cancer (694,000 deaths), breast cancer (521,000 deaths), and oesophageal cancer (400,000 deaths). The five most common sites of cancer in men in 2012 were lung, prostate, colorectum, stomach, and liver, and in women were at breast, colorectum, lung, cervix, and stomach cancer.

National Cancer Institute in Thailand reported that the number of people dying from cancer disease is still increasing every year. The fifteen leading site of cancer in male and female patients in 2012 were indicated in table 2.1 and 2.2, respectively.

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Table 2.1 The fifteen leading sites of cancer in male patient **:** 2012

Ordinal	Site	No.	%
1	Trachea, Bronchus and Lung	252	16.22
2	Colon and Rectum	230	14.80
3	Liver and Intrahepatic bile ducts	191	12.29
4	Lip and Oral cavity	115	7.40
5	Esophagus	99	6.37
6	Prostate gland	79	5.08
7	Nasopharynx	71	4.57
8	Non-Hodgkin's lymphoma	54	3.47
9	Larynx	44	2.83
10	Oropharynx	43	2.77
11	Stomach	39	2.51
12	Skin	36	2.32
13	Without specification of site	27	1.74
14	Mesothelial and Soft tissue	24	1.54
15	Tonsil	24	1.54

(NCI Thailand, 2015)

Table 2.2 The fifteen leading sites of cancer in female patient **:** 2012

Ordinal	**Site**	**No.**	**%**
1	Breast	939	39.74
2	Cervix uteri	340	14.39
3	Colon and Rectum	224	9.48
4	Trachea, Bronchus and Lung	155	6.56
5	Liver and Intrahepatic bile ducts	94	3.98
6	Ovary	87	3.68
7	Lip and oral cavity	73	3.09
8	Corpus uteri	71	3.00
9	Non-Hodgkin’s lymphoma	55	2.33
10	Thyroid gland	46	1.95
11	Skin	43	1.82
12	Stomach	37	1.57
13	Nasopharynx	29	1.23
14	Pancreas	19	0.80
15	Brain	18	0.76

(NCI Thailand, 2015)

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2.2 Causes and risk factors of cancer

2.2.1 External factors

It is believed that cancer is mostly rooted from the following external factors. For example carcinogen-contaminated food and drink such as aflatoxins in moldy foods. Aflatoxins are toxin produced by fungi that are found on agricultural crops such as corn, peanut and tree nut (Abnet 2007). Fungi can contaminate crops in the field during harvestation and storage (Lachenmeier 2009). Cooking methods that involve with grilling are also carcinogenic. Grilled meat, fish vegetable and other foods with intense heat over a direct flame results in fat dripping on the hot fire and yielding flames containing a number of polycyclic aromatic hydrocarbons (PAHs). These chemicals adhere to the surface of the food (Jagerstad and Skog 2005). Others include nitrosamine in preserved foods and food coloring agents. These are made from dye used in textile industry (Lijinsky 1999). Habits, such as smoking and drinking behaviors, are the major risk factors for oral and pharyngeal cancer (Blot et al. 1988). Ultraviolet ray in sunlight. People who are frequently exposed to strong sunlight, which has high amount of ultraviolet ray, are at risk of skin cancer (Armstrong and Kricker 2001). The risk of getting some types of cancer may be reduced by changes in a person's lifestyle.

2.2.2 Internal factors

Play a role with congenital genetic disorder that poses high risk of leukemia. Examples include immunodeficiency and malnutrition (*e.g.* lacking vitamin C). It is an antioxidant and plays a key role in collagen biosynthesis. High doses of vitamin C may slow the growth and spread of prostate, pancreatic, liver, colon, and other types of cancer cells (Block 1991).

2.3 Cancer biology

Cancer is a disease caused by alternation of normal cell into a cancer cell. Cancer arises from external and internal factors as a result of damaged DNA. Cancer cells separate themselves rapidly and become a tumor, and the normal cells eventually die because of the lack of sufficient blood. Cancer is divided into different categories depending on the organ the cancer cells are found: lung cancer, brain cancer, breast cancer, cervical cancer, leukemia, lymphoma, and skin cancer, for examples. The first cancer cell starts to divide producing daughter cells, and more and more cancer cells. They develop malignant characteristics including metastasis, immune system evasion, and induction of blood vessels formation (angiogenesis). Continuous cell division of cancer cells lead to the tumors. In solid tumors, blood vessels become structurally and functionally abnormal. This abnormality leads to heterogeneous blood flow which creates chronically hypoxic and acidic regions in the core of the solid tumor (Brown and Wilson 2004). Furthermore, the low pH microenvironment of cancer cells in the tumor core may prevent the active uptake of some anticancer drugs or some chemotherapies (Mahoney et al. 2003).

2.4 Types of cancers

As far as reported, cancer varies in more than 100 categories. Each type differs in severity. Types of cancer are shown in Table 2.3.

Table 2.3 Types of cancer

Cancer	Types of cancer
Cancers of Blood and Lymphatic Systems	Hodgkin's disease Leukemias Lymphomas Multiple myeloma, Waldenstrom's disease
Skin Cancers	Malignant Melanoma
Cancers of Digestive Systems	esophageal cancer, Stomach cancer Cancer of pancreas Liver cancer Colon and Rectal cancer, Anal cancer
Cancers of Urinary system	Kidney cancer Bladder cancer Testis cancer Prostate cancer
Cancers in women	Breast cancer Ovarian cancer Gynecological cancer Choriocarcinoma

Table 2.3 Types of cancer (Cont.)

Cancer	Types of cancer
Miscellaneous cancers	Brain cancer Bone cancer Carcinoid cancer Nasopharyngeal cancer Retroperitoneal sarcomas Soft tissue cancer Thyroid cancer

(Sakarkar and Deshmukh 2011)

2.5 Cancer treatment

Cancer treatment in Thailand is by surgery (operation to remove tumor and neighboring lymph nodes), radiation oncology (delivery of radiation beam to localized areas of cancer), chemotherapy (drug consumption or injection to kill cancer cells that spread through blood or lymph), and hormone therapy (strengthening immune system against certain drugs), or a combination of these. However, hormone therapy is still in its infancy. Further studies are necessary to confirm the effectiveness of this treatment when in coordination with chemotherapy (Pezzuto 1997).

Chemotherapy is chemical or drug used to cure of cancer. The chemical or drug which kill cancer cells also possessed side effects that some normal cells are also killed. Chemotherapy often result in side effects on normal cells, especially on those cells that differentiate such rapidly, as epithelial tissue cells in gastrointestinal tract, hair cells, and blood cells. Therefore, these cells are highly affected by

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chemotherapy. Frequently observed side effects from chemotherapy are such as hair loss, nausea, oral ulcers and reduction of blood cells. Side effects requiring doctor consultation are, such as fever and shivering, oral bleeding or ulcers, rash or other allergy, breathing difficulty, severe diarrhea or constipation and bleeding urine or feces. Different types of side effects depend on types of chemotherapy and patients' body reaction, thus, discovery of anticancer agents or novel drugs is desired. Which that drug should be effective against cancer cells and less toxicity to normal cells (Pezzuto 1997).

2.6 The use of natural product from plants as cancer therapy agent

In the present, scientists and researchers are studying about properties of herbs for therapy and the treatment of various symptoms and diseases, increasing chance in discovering of novel drug and increasing of alternative for defend of disease. Plants are one of the main sources of drugs and biologically active materials. Recently medicinal herbs are used by 80% of the people living in countryside as primary healthcare system (Sakarkar and Deshmukh 2011). Potential medicinal values have been studied chemically and pharmacologically, and only 1-10% out of the 250,000 – 500,000 plant species on earth (Verpoorte 2000). Even though the synthetic chemistry is currently used as a method to discover and manufacture drugs, the potential of bioactive plants to provide novel products for disease treatment and prevention is still enormous (Raskin et al. 2002). Plants-derived agents may have different mechanisms than conventional drugs, and could be of clinical importance in health care improvement (Eloff 1998). Plant materials might contain bioactive secondary metabolites that have the potential to treat different illnesses (Quiroga et al. 2001).

Such plant derived natural products are the main focus of many scientists to develop new medicine for various diseases such as cancer and microbial infection. Scientists are seeing for cures for cancer using natural pure compounds. Research them have been focused on the use of natural products from plants such as crude extracts or different phytochemicals in combination for cancer therapy. This trend is based upon the synergistic effect of the different plant metabolites in the crude extract, also multiple points of intervention of such extracts (Neergheen et al. 2010). This is one of the many faces of using plants in the quest of controlling different diseases. Since 1961, nine pure compounds extracted from plants were approved for use in cancer therapy in the United States. These agents are vinblastine, vincristine, navelbine, etoposide, teniposide, taxol, taxotere, topotecan, and irinotecan (Lee 1999). In an expanded study, anticancer properties of 187 plant species were assessed. Among them, only fifteen species have been used for clinical treatment of cancer (Kintzios 2007). It was observed that different plants contain different bioactive compounds. These variations were concerned with area, climate and mode of agricultural practice, in case they are not present in wild environment.

From the ethnopharmacological approach of Thai folk doctors for cancer treatment, a search for bioactive compounds against cancer cells has been reviewed in Table 2.4.

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Table 2.4 Medicinal plants used by Thai folk doctors in traditional preparations for cancer treatment

Botanical name	English/Thai name	Activity
Aloe vera L. (Liliaceae)	Aloe vera/ Vanhangjorakae	Cytotoxic
Bridelia ovate Decne (Euphorbiaceae)	Maka	Cytotoxic, Antimutagenic Antitumor
Citrus hystrix DC. (Rutaceae)	Kaffir Line/ Makrud	Antitumor
Curcuma zedoaria (Berg) Roscoe (Zingiberaceae)	Zedoary, white turmeric/ Kaminooy	Antitumor, Cytotoxic, Antimutagenic
Dioscorea membranacea Pierre (Dioscoreaceae)	Hua Khao Yen Tai	Cytotoxic
Dioscorea birmanica Burkill (Dioscoreaceae)	Hua Khao Yen	Cytotoxic
Eugenia jambos L. (Mytaceae)	Rose apple, Pomariosa/ Chompunamdogmai	Cytotoxic
Manihot esculentus Cranz (Euphorbiaceae)	Cassava/ Munsumpalung	Cytotoxic
Moringa oleifera Lamk (Moringaceae)	Horseradish-tree, Ben-oil tree, Drumstick-tree/ Marum	Antitumor. Cytotoxic

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Table 2.4 Medicinal plants used by Thai folk doctors in traditional preparations for cancer treatment (Cont.)

Botanical name	English/Thai name	Activity
Passiflora foetida L. (Passifloraceae)	Stinking Passion Flower/ Taosingto Long peper/	Cytotoxic
Piper chaba Hunter (Piperaceae)	Deeplee	Cytotoxic, Antitumor
Piper sarmentosum Roxb. (Piperaceae)	Thai Betel leaf/Chaa phluu	Cytotoxic
Piper interruptum Opiz.	Sakhane	Antitumor Cytotoxic
Plumbago indica L.	Rose-colore lead-wort/ Jedtamulplengdang	Antimutagenic Cytotoxic
Rhinacanthus nasutus (L.) Kurz (Acanthaceae)	White crane flower/ Thongpunchang	Antitumor
Smilax corbularia Kunth (Liliaceae)	Hua Khao Yen Neu	Cytotoxic Antitumor
Strychnos nux-vomica (Loganiaceae)	Vomit nut/Kod klakling	Cytotoxic
Zingiber officinale Roscoe (Zingiberaceae)	Ginger/Khing	Cytotoxic Antitumor
Zingiber zerumbet (L.) Roscoe ex Smith (Zingiberaceae)	Pinecone ginger, Shampoo ginger/Kratue	Antitumor

(Itharat and Ooraikul 2007)

2.7 Calcium signaling pathway

Calcium signaling pathway is one of pathways which has important roles in controlling cellular proliferation, T-cell activation, secretion, muscle contraction and neurotransmitter release in higher eukaryotes (Clapham 1995)

In the yeast Δ*zds1* strain *Saccharomyces cerevisiae*, the Ca^{2+} signal is implicated in the regulation of G_2/M cell cycle progression showed in figure 2.1.
In the yeast Δ*zds1* strain *S. cerevisiae* cells are grown in a medium containing high concentration of $CaCl_2$ which leads to the activation of the cellular Ca^{2+}-signaling pathways. The cell cycle regulation by Ca^{2+} is performed through the activation of the two parallel pathways: calcineurin pathway, and the Mpk1 MAP kinase cascade pathway. These two pathways cooperatively activate Swe1, a kinase protein. The SWE1 expression level is highly induced by Ca^{2+}. Swe1 kinase inhibits a G_2 form of the Cdc28 cyclin-dependent protein kinase by phosphorylating it at Tyrosine-19 and lead to delay entry into G_2 phase.

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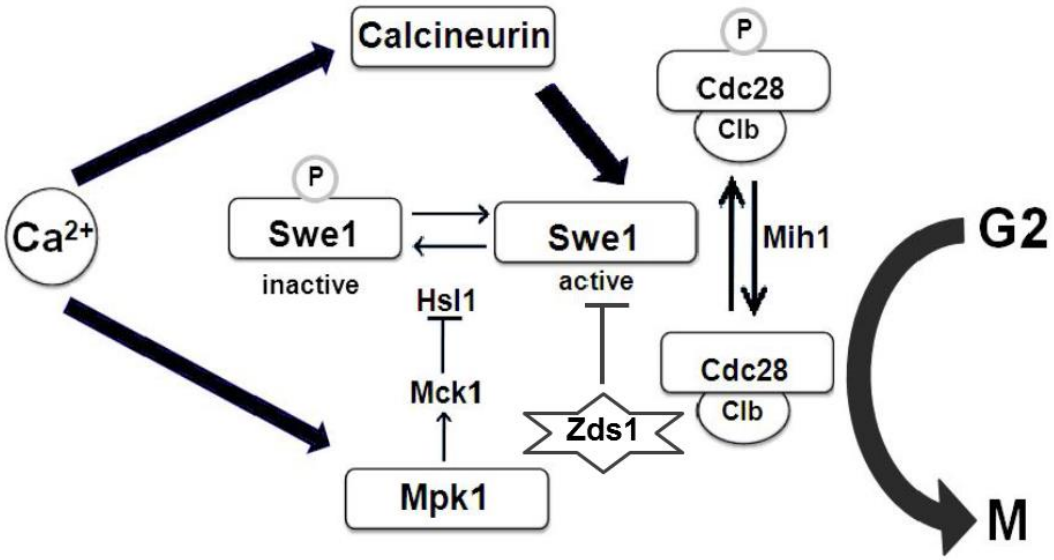

Figure 2.1 The Ca^{2+}-activated signaling pathways that regulated G_2 cell cycle progression in *S. cerevisiae*. The picture was modified from (Shitamukai et al. 2000)

Substances that inhibit calcium signaling pathway have the potential to develop into medicines. The inhibitor of calcium signaling pathway are expected to be of great pharmaceutical interest.

Previous studies of Ca^{2+} inhibitor were conducted in our laboratory using Δzds1 proliferation assay to screen for bioactive compounds from 74 crude ethanol extracts of Thai medicinal plants. Pakdeemai (2008) found that the *C. harmandiana* (Pierre) was one of the strong positive plant samples. Recently, Suauam (2011) found a coumarin compound, that was isolated from leave extract of *C. harmandiana* (Pierre), exerted a strong inhibitory activity on the Ca^{2+}-signal mediated cell-cycle regulation in the yeast *S. cerevisiae*.

2.8 *Clausena harmandiana* (Pierre)

C. harmandian (Pierre) is commonly known in Thai as Song faa dong or Song faa. It is a Thai herbal medicidal plant in family Rutaceae and can be found in the East, North-East and South of Thailand. It has different local names in each local such as Prong faa in Central of Thailand, Long faa in Udonthani province, Song faa dong in Loei province, Men in Chanthaburi province and Samui hom in Nakhonsithammarat province. The plant in genus Clausena got discovered and brought to use as a herb for treatment of several diseases in India such as diarrhea, kidney stone, uterine pain and pain symptom caused by liver and spleen (Chopra et al. 1956). The previous study on extracts from *C. anisata, C. heptaphylla, C. indica, C. wampi* and *C. pentaphylla* were shown effective to reduce spasmolytic activity (Patnaik and Dhawan 1982). However, little is known on *C. harmandiana*'s biological activity. Recently, it has been reported that it possesses some biological activity such as antibacterial activity (Maneerat et al. 2012).

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2.9 Characterization of *C. harmadiana* (Pierre)

C. harmandiana plant is a perennial shrub, with erected, greenish-brown and hairless stalk. The leaves are odd-pinnately and alternate. It contains about 3-7 leaflets. The shape of leaves are ovate-oblong and the end of leaves are acute. The leaves are 6.81-11.05 centimeters long and 2.82-5.32 centimeters wide. The leaves are dark green, lustrous, punctate and widespread of leaves when the leaves are shine through. It will pass to the next side. It is original word of Song faa dong. The vein is pinnately netted venation. Song faa dong is flowering in March to November. Bunch

of flowers are in panicle form. Flowers are small and yellowish-green. The pollen is yellow and fruit sets in April. The fruits are egg shaped (Chuakul et al. 2000).

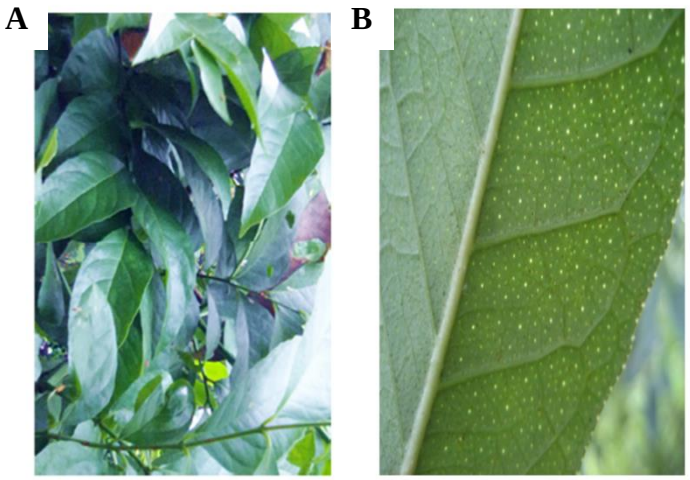

Figure 2.2 Morphology of stem (A) and leaves (B) of *C. harmandiana* (Pierre) (Morad 2013)

Figure 2.3 Bunch of fruit of *C. harmandiana* (Pierre) (Morad 2013)

2.10 Biological activities of *C. harmadiana* (Pierre)

Yenjai et al. (2000) discovered compounds that were extracted from *C. harmandiana* and identified four known compounds such as heptaphlline, clausine K, dentatin and clausarin. It was found that heptaphlline, dentatin, and clausarin showed antiplasmodial activity against to *Plasmodium falciparum*.

Thongthoom et al. (2010) studied biological activity of chemical constituents from *C. harmandiana*, and found that a coumarin, a ferulate and eight carbazoles that were extracted from root of *C. harmandiana* possess strong cytotoxicity against NCI-H187 (human lung cancer), MCF-7 (breast cancer) and KB (oral human epidermal carcinoma) cell lines.

Maneerat et al. (2012) reported that three new carbazole alkaloids, harmandianamines A, B and C, with fifteen known compounds were isolated from the twigs of *C. harmandiana* showed antibacterial activity against *Escherichia coli* TISTR 780, *Salmonella* Typhimurium TISTR 292, *Staphylococcus aureus* TISTR 1466 and methicillin-resistant *S. aureus* (MRSA).

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Songsiang et al. (2012) isolated nine carbazoles and three coumarins from *C. harmandiana* and detected antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and lipid peroxidation assay. Those extract showed cytotoxicity against cholangiocarcinoma, KKU-OCA17 and KKU-214 cell lines. In addition, they found that 7-Hydroxyheptaphylline and nordentatin showed strong antioxidant activity on lipid peroxidation and strong cytotoxicity against KKU-OCA17 and KKU-214 cell lines and also antioxidant activity. The results showed that these compounds might be promising leads to the development of cytotoxic agents.

Maneerat et al. (2013) found a new phenylpropanoid derivative, harmandianone, four known compounds (verimol B, (E)-3-(2-hydroxy-4-methoxy phenyl) propanoate, (E)-methyl p-coumarate, and (E)-5- methoxy-2-(prop-1-enyl) phenol) in the acetone extract from fruits of *C. harmandiana*. They showed antibacterial activity against *E. coli* TISTR 780, methicillin-resistant *S. aureus* SK1, *S.* Typhimurium TISTR 292 *and S. aureus* TISTR1466.

2.11 Coumarin compound

Coumarin is classified as a member of the benzopyrone family of compounds, all of which consist of a benzene ring joined to a pyrone ring (Jain and Joshi 2012). It is a plant-derived natural product known for its pharmacological properties such as anti-inflammatory (Huang et al. 2012), anticoagulant, antibacterial, antifungal (Wang et al. 2009), antiviral (Patil et al. 1993), anticancer (Luo et al. 2011), antihypertensive (Crichton and Waterman 1978), antitubercular (Chiang et al. 2010), anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant, and neuroprotective properties (Venugopala et al. 2013). In this study, CHA-01 is a coumarin compound extracted from leave of *C. harmandiana* (Pierre).

CHAPTER III

Material and Methods

3.1 Instruments used in this thesis

1. 37°C Incubater (Thermo Scientific, USA)

2. 37°C CO_2 Incubater (Thermo Scientific, USA)

3. 96 well plate (low binding) (NUNC, Sweden)

4. Adhesive optical sealing film 80×120 mm (BIONEER, South Korea)

5. Agarose gel electrophoresis Mini gel electrophoresis system (Mupid-ex, Japan)

6. Alcohol burner

7. Autoclave MLS 3020 (Sanyo, Japan)

8. Beaker (Pyrex, USA)

9. Bench-top centrifuge 2600 (Denvelle, Germany)

10. Bottle for prepare media (tissue culture) 100 ml (Corning Incorporation, USA)

11. Centrifuge tubes 15 and 50 ml (Corning Incorporation, USA)

12. Cover slips (Nissho Nipro, Japan)

13. Cryotube (Corning Incorporation, USA)

14. Deep freezer ULT 1786 (Forma Scientific, USA)

15. Deep freezer MDF-U332 (Sanyo electric, Japan)

16. Disposable syringe (Nissho Nipro, Japan)

17. DNA Thermal Cycle 2400 (Bio-Rad, USA)

18. Erlenmeyer Flasks 125, 250 and 500 ml (Corning Incorporation, USA)

19. *Exicycler*™ 96 Quantitative Real-Time PCR System (BIONEER, South Korea)

20. Filter paper (Whatman, England)

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21. Gel Documentation and Quantity one program Version 4.4.1 (Bio-Rad, USA)
22. Haemacytometer (Mettler Toledo, Switzerland)
23. Hot air oven UE600 (Memmert, Germany)
24. Inverted microscrope (Olympus, USA)
25. Laminar flow Clean model V4 (LAB Service, Thailand)
26. Measuring cylinder (Pyrex, USA)
27. Microcentrifuge tubes 1.50 ml (Axygen Scientific, USA)
28. Microcentrifuge tubes, Low binding polymer technology (Sorenson, USA)
29. Micropipette P10 P20 P100 P200 P1000 and P5000 (Gilson, France)
30. Microplate reader Elx 800 (Bio-tek instrument, USA)
31. Microscope slides (Nissho Nipro, Japan)
32. Microwave oven (Samsung, Korea)
33. Nanoscale LC-MS for Synapt HDMS system, NanoEquity System (Waters Corp. Milford, MA)
34. Parafilm (Parafilm®M, USA)
35. PCR tube 200 µl (Corning Incorporation, USA)
36. PCR tube 0.2 ml Opaque white 8-strip (BIONEER, South Korea)
37. pH meter S-20K (Mettler-Toledo, Switzerland)
38. Pipette aid (Drummond, USA)
39. Power supply for electrophoresis (Atto, Japan)
40. Refrigerator Tiara (Mitsubishi Electric, Thailand)
41. RNase-free tip 2, 10, 100, 1000 µl (Axygen Scientific, USA)
42. Rotary vacuum evaporator N-1NW (Eyela, Japan)
43. Seropipettes 1, 5 and 10 ml (Pyrex, USA)

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44. SDS-polyacrylamide gel electrophoresis (Atto, Japan)

45. Sonicator RK 100 (Bandelin, Germany)

46. Syringe filter CA-CN 13 mm 0.22 μM (Restek, Thailand)

47. Thermo-block MylabTHThermoBlock SLTDB-120 (Seoul in Bioscience, Korea)

48. Tissue culture flask 25, 75 cm^3 (NUNC, Denmark)

49. Tissue culture plate 6, 12, 24 and 96 well (NUNC, Denmark)

50. Vortex mixer Geniell G-560E (Scientific Industries, USA)

51. Water bath shaker NST 2000 (Eyela, Japan)

52. Weighing machine PB3002 (Mettler-Toledo, Switzerland)

3.2 Chemical

1. 5-Bromo-2'-deoxyuridine (BrdU) (Merck, Germany)

2. Absolute ethanol (Lab Scan analytical science, USA)

3. Absolute methanol (Merck, Germany)

4. Acetic acid (Lab Scan analytical science, USA)

5. Acetone (Lab Scan analytical science, USA)

6. Acetronitrile (J.J.Baker, White group, USA)

7. Acrylamide (GE healthcare, Sweden)

8. Agarose powder (Prondisa, Spain)

9. Ammonium bicarbonate (Sigma, USA)

10. Ammonium persulfate (Plusone, Amersham Bioscience, Sweden)

11. ApopNexin™ FITC (Merck, Germany)

12. Bovine serum albumin (Amersham Bioscience, Sweden)

13. Chloroform (Lab Scan analytical science, USA)

14. Coomassie Brilliant Blue R-250 (Sigma, USA)

15. Copper sulfate (Fisher Scientific, UK)

16. DNA ladder 100 bp and 1 kb (New England Biolabs inc., England)

17. Diethylpyrocarbonate (DEPC) (Sigma, USA)

18. DNase I (Fermentas, Canada)

19. Dithiothreitol (DTT) (USB corporation, USA)

20. Dimethyl sulfoxide (DMSO) (Amresco, USA)

21. dNTPs mix (Fermentas, Canada)

22. EGTA (Ethylene-bis (oxyethylenenitrilo) tetraacetic acid tetrasodium) ($C_{14}H_{20}N_2O_{10}Na_4$) (Sigma, USA)

23. Fetal Bovine Serum (FBS) (Hyclone, UK)

24. Ficoll-Hypaque (GE Healthcare Bio-Sciences, UK)

25. FITC Mouse Anti-BrdU Set (BD Pharmingen, USA)

26. FK506 (Sigma, USA)

27. Follin-Ciocalteu reagent (Sigma, USA)

28. Formaldehyde (Sigma, USA)

29. Formic acid (Fluka, Germany)

30. Glycerol (Carlo ERBA, France)

31. Hydrochloric acid (HCl) (LAB-SCAN, Ireland)

32. Hydrogen peroxide (Merck, Germany)

33. Iodoacetamide (IAA) (GE healthcare, Sweden)

34. Isopropanol (Merck, Germany)

35. KAPA SYBR® FAST Master Mix ABI Prism™ (Kapa Biosystems, USA)

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36. Magnesium chloride ($MgCl_2$) (Merck, Germany)
37. Magnesium sulfate ($MgSO_4$) (Merck, Germany)
38. Methanol (Lab Scan analytical science, USA) (Bio Basic inc, Canada)
39. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Bio Basic inc, Canada)
40. Penicillin (General Drugs House, Thailand)
41. Potassium chloride (KCl) (Merck, Germany)
42. Potassium di-hydrogen phosphate (KH2PO4) (Merck, Germany)
43. Propidium iodide (Sigma, USA)
44. Protease inhibitor (Sigma, USA)
45. Random hexamer (Fermentas, Canada)
46. Reverse transcriptase (Fermentas, Canada)
47. Ribonuclease A (Sigma, USA)
48. Ribonuclease inhibitor (Fermentus, Canada)
49. RPMI 1640 (Hyclone, UK)
50. Streptomycin (M & H Manufacturing, Thailand)
51. SDS (sodium dodecyl sulfate), ($C_{12}H_{25}OSO_3$) (Sigma, USA)
52. Sodium hydroxide (NaOH) (Merck, Germany)
53. Sodium di-hydrogen phosphate (Na_2HPO_4) (Merck, Germany)
54. Sodium phosphate (NaH_2PO_4) Merck, Germany
55. Sodium carbonate (Na_2CO_3) (Merck, Germany)
56. Taq DNA polymerase (New England Biolabs inc., USA)
57. TEMED (N,N,N,N-Tetramethyethylenediamide) (Bio Basic inc, Canada)
58. Triton X-100 (Sigma, USA)

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59. Trizol reagent (Invitrogen, USA)

60. Trypsin (Promega, USA)

61. Trypan blue 0.5% w/v (Biochrom AG, Germany)

62. Tris buffer pH 8.8 (Preparation is described in appendix)

63. Tris buffer pH 6.8 (Preparation is described in appendix)

64. Tween 20 (Sigma, USA)

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3.3 Primer sequences and conditions used in quantitative real-time PCR (qPCR)

Table 3.1 Primer sequences and conditions used in quantitative real-time PCR (qPCR)

Primer	Nucleotide sequence	Annealing temp. (°C)	Product Size (bp)	Reference
MCM4	Forward: GCATCTATCGAGCTGTGCCT Reverse: GCAGACGTTTTGCATCCGTT	53	114	This study
NPM1	Forward: CGGTTGTGAACTAAAGGCCG Reverse: TTTGCACCAGCCCCTAAACT	51	105	This study
POLD3	Forward: ATGGCGGACCAGCTTTATCT Reverse: AGCATCTGTTTGGCCTGGTT	53	122	This study

Table 3.1 Primer sequences and conditions used in quantitative real-time PCR (qPCR) (Cont.)

Primer	**Nucleotide sequence**	**Annealing temp. (°C)**	**Product Size (bp)**	**Reference**
PRKACB	Forward: GGTGGAGAGCGTGAAAGAGT Reverse: ACTGTTCAGTGGCTTTGTGT	51	170	This study
FADD	Forward: GATTGGAGAAGGCTGGCTCG Reverse: GATTCTCAGTGACTCCCGCA	58	111	This study
GAPDH	Forward: TGCACCACCAACTGCTTAGC Reverse: GGCATGGACTGTGGTCATGAG	55	87	(Yuan et al. 2004)

3.4 Source of CHA-01

A pure coumarin, CHA-01, isolated and purified from leave extract of *C. harmandiana* (Pierre) was collected from Khao Hin Son National Park, Phanom Sarakham, Chachoengsao Province. The procedure for extraction and purification of *C. harmandiana* (Pierre) was performed at laboratory of Assoc. Prof. Dr. Boonek Yingyongnarongkul, Department of Chemistry, Faculty of Science, Ramkamhaeng University, Thailand, using column chromatography techniques. The solution of CHA-01 was stocked in dimethyl sulfoxide (DMSO).

3.5 CHA-01 dissolving method

Addition of DMSO little by little into a microfuge tube containing 3.98 mg of CHA-01 and vortexing until homogeneous was obtained. Then adjusting the final volume to 100 µl by DMSO to obtain CHA-01 final concentration of 100 mM (Molecular weight 398 g/mol). To see the effect of CHA-01 on cell culture, CHA-01 was dissolved in RPMI by adding RPMI, as solvent into the new tube, and then added CHA-01 gradually. Then vortexed until homogeneous was obtained. CHA-01 was added until final concentration was reached. Using this dissolving method, CHA-01 will not crystalize or aggregate in solution.

3.6 Cancer cell lines of human

Jurkat (Human acute T cell leukemia) ATCC number CRL-2063, THP-1 (Human acute monocytic leukemia) ATCC number TIB-202, KATO III (Human gastric carcinoma) ATCC number HTB-103 were obtained from Assoc. Prof. Dr. Tanapat Palaga, Department of Microbiology, Faculty of Science, Chulalongkorn

University, Thailand. Normal human blood cells were obtained from the Thai Red Cross Society, Bangkok, Thailand.

3.6.1 Cell lines and cell cultivation

Jurkat T cell (Human acute T cell leukemia), THP-1 (Human acute monocytic leukemia) and KATO III (Human gastric carcinoma) were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 10^6 U/ml penicillin and 500 mg/ml streptomycin. Cells were cultured at 37°C in 5% CO_2 containing atmosphere.

3.6.2 Cell preservations

Jurkat T cell, THP-1 and KATO III were collected from culture flasks and centrifuged at 200 × *g* for 5 min. Cell pellets were resuspened in 1 ml of freezing media (10% (v/v) DMSO in RPMI-1640 complete media) and transferred to cryogenic vials. Cell were immediately stored at -80°C.

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3.6.3 Cell preparation

Jurkat T cell, THP-1 and KATO III were collected from culture flasks and centrifuged at 1,000 rpm for 5 min. The supernatant was discarded and the cell pellets were resuspended in RPMI-1640 complete media. Viable cells were diluted in trypan blue dye and counted by using a hemacytometer.

3.7 MTT Proliferation assay

To detect cytotoxic effect of CHA-01, viability of Jurkat T cell, KATO III and THP-1 cell lines treated with various concentrations of CHA-01 was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Won et al. 2006). The MTT assay is based on the conversion of yellow tetrazolium salt MTT into purple formazan crystals by living cells, which indicates mitochondrial activity.

Briefly, the test cells were seeded at a final concentration of 5×10^4 cells/ml in a 96-well plate and incubated overnight for adherent cell (KATO III) and then treated with various concentrations of CHA-01 (0.001, 0.01, 0.1, 1, 10, 100 µM) dissolved in RPMI-1640. For non-adherent cell lines (Jurkat T and THP-1), cells were directly incubated with varying concentrations of CHA-01. For a control experiment, cells were treated with DMSO and incubated at 37°C in the atmosphere containing 5% CO_2 for 1 or 4 days. After 1 or 4 days of incubation at 37°C in the atmosphere containing 5% CO_2, 10 µl of MTT solution (5 mg/ml) was added to each well and the suspension was further incubated for 4 h. Then, 100 µl of 0.04 N HCl in isopropanol was added to dissolve the colored formazan crystal produced from MTT conversion. Optical density was measured at 540 nm by a microplate reader (ELx800 Bio-tek instrument, USA). Percent of cell viability was calculated using the following formula :

$$\% \text{ Cell viability} = (\text{OD test} - \text{OD blank}) \times 100 / (\text{OD control} - \text{OD blank})$$

OD test: value of optical density of cell treated with CHA-01 or DMSO.

OD control: value of optical density of cell.

OD blank: value of optical density of medium.

3.8 Preparation of human normal white blood cells and MTT Proliferation assay

Human normal white blood cells were isolated from whole blood by density gradient centrifugation using Ficoll-Hypaque following the manufacturer's instruction. The cytotoxic effect of the CHA-01 on human normal white blood cells was analyzed by MTT assay (Won et al. 2006) as previously described in section 3.7.

3.9 Hemolytic activity of CHA-01 on human red blood cell

The hemolytic activity of CHA-01 on human red blood cell was tested as described by Arpornsuwan et al. (2014). Human red blood cells were washed three times with PBS and then 1 % of cell suspension was diluted in phosphate-buffered saline (PBS). CHA-01 was diluted in PBS by 10-fold dilution with concentrations ranging from 100, 10, 1, 0.1 and 0.01 μM. Human red blood cells were seeded at a final concentration of 5×10^4 cells/ml in a 96-well plate and then treated with various concentrations of CHA-01 and then incubated at 37°C in the atmosphere containing 5% CO_2 for 24 h. The optical density of supernatant was measured at 540 nm. The PBS solution was used as a negative control and 1% Triton X-100 was used as a positive control. The % hemolysis was calculated as follow:

$$\% \text{ Hemolysis} = [(\text{OD CHA-01 treated} - \text{OD negative control}) / (\text{OD positive control} - \text{OD negative control})] \times 100$$

3.10 Cell cycle analysis by Flow cytometry

A total of 2.5×10^5 cells/well of Jurkat T cells were plated onto 24-well plates. The CHA-01 was added into the wells to obtain final concentrations of 0.5, 1 and 2 µM. The FK506 was added into the wells to obtain final concentrations of 1, 2 and 4 µM. The CsA was added into the wells to obtain final concentrations of 3, 6 and 12 µM and the plates were incubated at 37°C in the atmosphere containing 5% CO_2 for 24 h. The cells were centrifuged at 4200 × *g* for 5 min. The pellets were rinsed twice with phosphate-buffered saline (PBS) and fixed using cold 70% ethanol for overnight at 4°C. Then, the cells were centrifuged at 4200 × *g* for 5 min and incubated with 10 mg/ml RNase A at 37°C for 30 min. Cells were then stained with 1 mg/ml propidium iodide for 30 min at 37°C in the dark. The samples were analyzed by a flow cytometer (Cytomics FC500, Beckman Coulter, USA) set at FL3.

3.11 Investigation of apoptosis induced by CHA-01

A total of 2×10^6 cells/well of Jurkat T cells were plated onto 12-well plates. The CHA-01 was added into the wells to obtain final concentration of 1 µM and the plates were incubated at 37°C in the atmosphere containing 5% CO_2 for 6, 12 and 24 h. Cells were harvested into a chilled culture tube and spin down at 400 x *g* for 5 min and then the supernatant was aspirated. Cells were washed twice with 1 ml ice-cold PBS, spin cells and the supernatant was removed. Cells were resuspended in ice-cold 1X binding buffer at a concentration of 10^6 cells/ml. Two hundred (200) µL cell suspension was added with 3 µl of the annexin conjugate ApopNexin™ FITC. After that, 2 µl of 100X PI was added to ApopNexin™ FITC labeled cells. Cell suspension was mixed and incubated for 15 min at room temperature in the dark.

Samples were analyzed by a flow cytometer. ApopNexin™ FITC bound cells and PI stained cells were determined by generating a FITC (FL1) vs. PI (FL3) dot plot.

3.12 DNA synthesis inhibition analysis

Cells were plated at a density of 2×10^6 cells/well of Jurkat T cells. The CHA-01 was added into wells to obtain final concentration of 1 µM and 10 nM camptothecin or 1 µM gemcitabine was added into wells and incubated at 37 °C in 5% CO_2 containing atmosphere for 24 h. BrdU was added into the wells to obtain final concentration of 10 µM and incubated at 37°C in the atmosphere containing 5% CO_2 for 1 h. Cells were washed with PBS and fixed with 70% ethanol at 4°C for at least 4 h. Samples were washed with PBS and incubated with 2N HCl at room temperature and washed twice with PBS containing 0.5% Tween20 and incubated for 45 min with 20 µl anti-BrdUrd-FITC, then washed twice with PBS. Cell were incubated for 30 min with 100 µg/ml RNase, 10 µg/ml PI and analyzed by a flow cytometer.

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3.13 Proteomic analysis of CHA-01 treated Jurkat T cells

3.13.1 Protein extraction

A total of 1×10^6 cells/well of Jurkat T cells were plated onto 6-well plates. The CHA-01 was added into the wells to obtain final concentration of 1 µM and the plates were incubated at 37°C, 5%CO_2 containing atmosphere for 0, 15 and 60 min. Cells were harvested into a chilled tube and were centrifuged at 10,000 × g for 15 min, then the supernatant was removed. Pellets were rinsed with 1 ml cold phosphate-buffered saline (PBS) and lysed with addition of 40 µl cold RIPA buffer, mixed a few

times by micropipette, vortexed and centrifuged at 10,000 × g for 15 min. The supernatant was transferred to a new tube, mixed well with 2 volumes of cold acetone and incubated overnight at -20ºC to precipitate proteins. The mixture was centrifuged at 10,000 × g for 15 min and the supernatant was discarded. The pellet was dried in a Speedvac and resuspended in 40 µl of 0.5% SDS, protein concentration was determined and the crude protein extracts were stored at -80^{o}C prior to use.

3.13.2 Determination of protein concentration by Lowry method

Protein concentration was determined by Lowry method (1951). Protein precipitates were resuspended in 40 µl of 0.5% SDS. The absorbance at 690 nm (OD_{690}) was measured and the protein concentration was calculated using standard curve plotted between OD_{690} on Y-axis and BSA concentration (µg/ml) on X-axis.

3.13.3 Protein separation by SDS-PAGE

Total protein sample of 30 µg were loaded onto 12.5% SDS-polyacrylamide gel. The equal volume of protein samples were mixed with 5X sample buffer and boiled for 5 min before loading onto the 12.5% SDS-PAGE following Laemmli's method (1970). Electrophoresis was performed in SDS electrophoresis buffer until the tracking dye reached the bottom of the gel. After the electrophoresis had finished, gels were stained with Coomassie Brilliant Blue R-250.

3.13.4 In-gel digestion

Protein bands were excised. Gel plugs were placed in a 96 well plate and gels were destained with 25 mM ammonium bicarbonate (NH_4HCO_3) in 50% methanol. Steriled water was added (200 µl) and then shaked for 5 min at room temperature. Water was removed and 200 µl of 100% Acetonitrile (ACN) was added, shaked for 5 min at room temperature, then the ACN was removed. The gel plug was allowed to dry at room temperature. To reduce disulfide bond, gel was immersed in 20 µl of 10 mM dithiothreitol (DTT) in 10 mM NH_4HCO_3 per well. Then the gel was incubated at 56^{o}C for 1 h. After that the solution was removed. Then 30 µl of 100 mM iodoacetamide (IAA) in 10 mM NH_4HCO_3 (fresh preparing) was added and kept in the dark at room temperature for 1 h. The solution was removed and 200 µl of 100% ACN was added followed by shaking at room temperature for 5 min. Then the ACN was removed and this step was repeated once more. The gels were allowed to dry for 15 min and were added with 20 µl of 10 ng/µl trypsin and were incubated at 37^{o}C for overnight. The solution was transferred to a new 96 well plate and 30 µl of ACN was added and followed by shaking at room temperature before transferring. Furthermore peptide was extracted using 30 µl of 50% ACN in 0.1% formic acid and was shaked at room temperature for 10 min and then the extracted peptide solution were pooled together in the same plate . The extracted solution was dried at in 40^{o}C for overnight and tryptic peptides were kept at -80^{o}C.

3.13.5 LC-MS/MS analysis

The dried extracted peptides were resuspended with 12 µl of 0.1% formic acid in LC-MS grade water and transferred to low binding microcentrifuge tubes. Solution was centrifuged at 10,000 rpm for 10 min and transferred to vial tubes. LC-MS/MS analysis of digested peptide mixtures was performed using a Waters SYNAPT™ HDMS™ system. The resuspended peptide was injected onto the RP analytical column packed with a 1.7 µm Bridged Ethyl Hybrid (BEH) C18 material (Waters). Peptides were eluted with a linear gradient from 2% to 40% acetonitrile developed over 20 minutes at a flow rate of 350 nl/min. This was followed by a 15 min period of 80% acetonitrile to clean the column before returning to 2% acetonitrile for the next sample. The effluent samples were electrosprayed into a mass spectrometer (Synapt HDMS). MS/MS data of peptides were analyzed by DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare (Johansson et al. 2006, Thorsell et al. 2007)). The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix Science, London, UK (Perkins et al. 1999)) against NCBInr database. Data normalization and quantification of the changes in protein abundance between the control and treated samples were performed and visualized using *MultiExperiment Viewer* (Mev) software version 4.6.1 (Howe et al. 2010). Biological process function was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) (Kanehisa et al. 2014) and UniProt database (http://www.uniprot.org) to search for cellular functions interactions among proteins in the cell.

3.14 Quantitative real-time RT-PCR

3.14.1 RNA extraction

Jurkat T cells (1×10^6 cells/well) were plated onto 6-well plates. Cells were treated in the presence or absence of 1 μM CHA-01 and the plates were incubated at 37°C, 5%CO_2 containing atmosphere for 0, 15 and 60 min, respectively. DMSO was used as control. Total RNA was isolated by using Trizol reagent 1 ml /well. The sample were extracted with 200 μl of chloroform and virgorously mixed for 15 sec and incubated at room temperature for 2-3 min. After centrifugation for phase separation at 12000 × *g* at 4°C for 15 min, the aqueous phase was carefully collected and gently mixed with 400 μl of isopropanol. The sample were incubated at room temperature for 10 min to precipitate RNA, then centrifuged at 12000 × *g* at 4°C for 10 min. Supernatants were removed and RNA pellet were washed with 1 ml of cold 75% ethanol in 25% DEPC. The samples were centrifuged at 7500 × *g* at 4°C for 5 min. RNA pellets were air dried for 10-30 min. RNA pellets were then resuspended in 10-15 μl of DEPC water and incubated at 60°C for 10 min. RNA samples were stored at -80°C until use.

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3.14.2 Removal of genomic DNA from RNA preparation

Total 1 μg of RNA was mixed with 1 μl of 10X reation buffer with $MgCl_2$, 1 U of DNase I and adjusted the volume to 10 μl by DEPC water. The mixtures were incubated at 37°C for 30 min. The mixtures were added with 1 μl of 50 mM EDTA and incubated at 65°C for 10 min. RNA samples were stored at -80°C until use.

3.14.3 cDNA synthesis by reverse transcription

Total RNA, 1 µg, was used to synthesize cDNA. Total RNA was mixed with 1 µl of 0.2 µg/µl of random hexamer and adjusted the volume to 12.5 µl by DEPC water. The mixtures were heated at 65°C for 5 min. The PCR master mix was prepared by mixing 4 µl of 5X reverse transcriptase buffer, 2 µl of 10 mM dNTP mix and 0.5 µl of 40 U/µl of RNase inhibitor. After heating the RNA mixtures, the master mix was added to the mixtures and finally 200 U of reverse transcriptase per reaction was added to the mixtures. The reactions were performed by BIO RAD T100™ Thermal Cycler at 25°C for 10 min, 42°C for 60 min and 70°C for 10 min. The cDNA was stored at -20°C until use.

3.14.4 Quantitative polymerase chain reaction (qPCR)

Standard curves representing 10^3–10^8 copies (in triplicate) of recombinant plasmids of *NPM1, MCM4, MCM7, POLD3, PRKACB, MAD1L1* and the internal control, *GAPDH* inserted in pGEMT vector were constructed. qPCR was performed by using KAPA SYBR® FAST Master Mix ABI Prism™. Total reaction volume of 10 µl containing: 100 ng of cDNA, 10 µM forward primer and reverse primer and PCR grade water were prepared. The specific primers used to amplify each target genes were as followed (Table 3.1). The reactions were performed in *Exicycler*™ 96 Quantitative Real-Time PCR System (BIONEER, South Korea) by conditions as follows: 95°C for 2.30 min, followed by 40 cycles of 95°C for 30 sec, annealing temperature of each gene indicated in Table 3.3 for 30 sec, 72°C for 30 sec and 25°C for 1 min and store at 8°C. The relative expression of mRNA levels was calculated.

CHAPTER IV
RESULTS

Suauam (2011) explored biological activity of CHA-01, a coumarin compound extracted from leave of *C. harmandiana* (Pierre) Guillaumin. It exerted a strong inhibitory activity on the Ca^{2+}-signal mediated cell-cycle regulation in the yeast *S. cerevisiae*. Furthermore, CHA-01 was found to inhibit IL-2 production by stimulated Jurkat T cells at the transcriptional level. CHA-01 also did not cause acute cytotoxic effects against Jurkat T cell (up to 10 mM). In this study, the anti-proliferative and acute or chronic cytotoxicity effect of a coumarin, CHA-01, from *C. harmandiana* on various human cancer cell lines were evaluated. More details characterization of the coumarin's role on the cell cycle progression, induction of apoptosis and DNA synthesis inhibition in cancer cell lines were also investigated. The study of proteins involving in anti-proliferation activity in cancer cell lines affected by the CHA-01 were investigated for molecular insights.

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4.1 The effect of CHA-01 on the anti-proliferation activity on various human cancer cell lines

CHA-01 is a coumarin compound extracted from the leaves of *C. harmandiana*. Coumarin is a plant-derived natural product known for its pharmacological properties such as anti-inflammatory (Huang et al. 2012), anticoagulant, antibacterial, antifungal (Wang et al. 2009), antiviral (Patil et al. 1993), antihypertensive (Crichton and Waterman 1978), antitubercular (Chiang et al. 2010), anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant, and neuroprotective properties (Venugopala et al. 2013). In addition, coumarin have been found to exhibit anticancer activity (Luo et al. 2011) in various cancer cell lines such as LNCaP, PC-3 (Prostate carcinoma), ACHN (Renal cell carcinoma), H727 (Malignant carcinoid), HS-Sultan (Myeloma (IgG)) cell lines) (Marshall et al. 1994), human lung carcinoma cells (Lopez-Gonzalez et al. 2004), human cervical cancer HeLa Cells (Chuang et al. 2007). The role of CHA-01 on anti-cancer activity has not yet been reported.

To detect cytotoxic effect of CHA-01, viability of Jurkat T cell, KATO III and THP-1 cell lines treated with various concentrations of CHA-01 was analyzed by the MTT assay. Each of the cancer cell lines was treated with different dosages (from 0.001-100 μM) of CHA-01 for 1 day and 4 days. CHA-01 showed cytotoxic effects against Jurkat T cell, KATO III and THP-1 compared to the control (DMSO). The inhibition of cell proliferation was in a dose- and a time-dependent manner. CHA-01 did not show acute cytotoxic effects against Jurkat T cell up to 10 μM, KATO III at least 100 μM, THP-1 up to 1 μM. (Fig. 4.1, Left panel). After 4 days incubation with

CHA-01, all 3 cell lines showed a dramatically decrease in cell viability with IC_{50} values of 0.67 ± 0.01, 1.06 ± 0.07 and 1.05 ± 0.01 μM in Jurkat, KATO III and THP-1, respectively. Jurkat T cell line was the most sensitive to the CHA-01 among the three cell lines tested. It was chosen for further studies.

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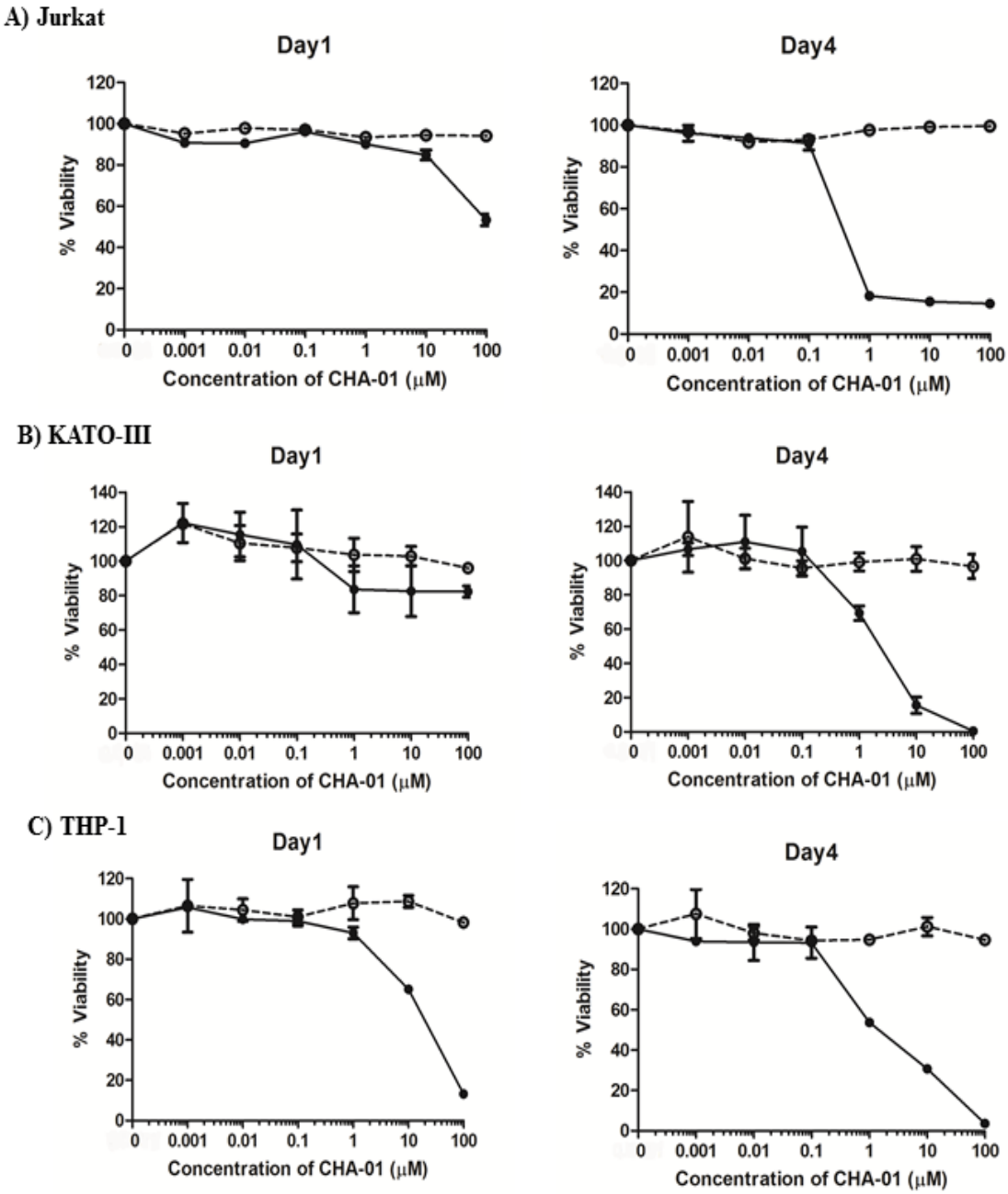

Figure 4.1 Cytotoxicity of CHA-01 on human cancer cell lines. Cytotoxicity was assessed using MTT assays. Cells of Jurkat (A), KATO III (B) and THP-1 (C)) were treated with various concentrations of CHA-01 (●) compared to those treated with DMSO (○) for 1 day and 4 days, respectively. The data at each time point represent mean ± SD from triplicate experiments.

4.2 The cytotoxic effect of CHA-01 on normal human white blood cells

Since Jurkat-T-lymphocytic leukemia cells showed the most pronounced cytotoxic effect when treated with CHA-01, it was questionable whether this cytotoxic effect could be observed in normal human white blood cells or not. Normal whole blood of blood group O, A and AB were obtained from the Thai Red Cross Society, Bangkok, Thailand.

They were separated from whole blood and then were treated with different dosages (from 0.001-100 μM) of CHA-01 for 1 and 4 days. Interestingly, CHA-01 showed much less toxicity to normal white blood cells (group O, A, AB) than the Jurkat T cells. On day 4, percentage of viability decreased to lower than that of day 1 due to life span of white blood cells (Kline and Cliffton 1952). The tested normal white blood cells (group O, A and AB) showed no cytotoxic to CHA-01 up to the dosage of 10 μM (Fig. 4.2, Right panel)

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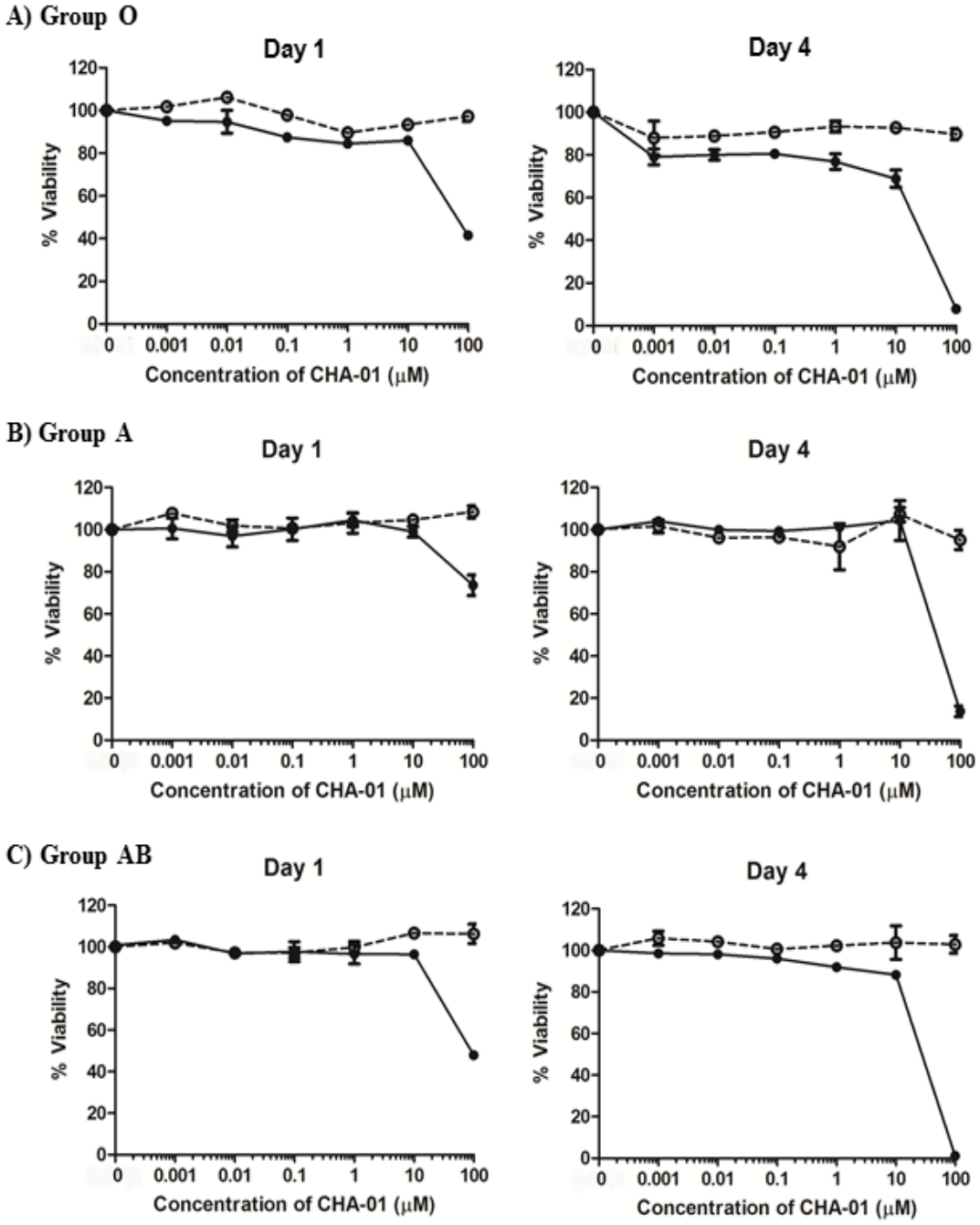

Figure 4.2 Cytotoxicity of CHA-01 on normal human white blood cells. Cytotoxicity was assessed using MTT assays. Cells (Group O (A), Group A (B) and Group AB (C)) were treated with various concentrations of CHA-01 (●) compared to the control (DMSO) (○) for 1 day and 4 days, respectively. The data at each time point represent mean ± SD from triplicate experiments.

4.3 Evaluaton on Hemolytic activity effect of CHA-01 against human red blood cells

CHA-01 showed much less toxicity to normal white blood cells than that of leukemia cells. We further investigated the cytotoxic effect against human red blood cells. The results showed that at concentration of CHA-01 up to 100 μM, no cytotoxic effect on human red blood cells was detected. That the percentage of hemolysis was nearly closed to control treatment (DMSO which is the concentration for dissolving CHA-01). Therefore, CHA-01 did not show cytotoxicity especially hemolytic activity on human red blood cells (Table 4.1).

Table 4.1 No hemolytic activity of CHA-01 against normal red blood cells

Concentration of CHA-01 (μM)	**Hemolysis (%)**	
	CHA-01	**DMSO***
100	2.14 ± 0.00	2.27 ± 0.17
10	1.29 ± 0.52	2.14 ± 1.04
1	1.04 ± 0.52	1.04 ± 0.17
0.1	1.41 ± 1.04	1.04 ± 0.52
0.01	0.55 ± 0.17	1.29 ± 0.17

The data are expressed as the mean ± SD from three different preparations (n = 3).

*DMSO was the same concentration for dissolving CHA-01

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4.4 The cytotoxic effect of FK506 and Cyclosporine A (CsA) on Jurkat T cell

The previous study showed that CHA-01 possesses calcineurin inhibiting activity (Suauam 2011). Anticancer activity of FK506 and Cyclosporine A a known calcineurin inhibitor were investigated.

Jurkat T cells were treated with different dosages (from 0.001-100 μM) of FK506 and CsA for 1 and 4 days. The cell viability was determined by MTT assay. FK506 did not show acute cytotoxic effect against Jurkat T cells, up to 10 μM and IC_{50} value of 23.00 ± 3.61 μM was determined after incubated for 1 day. After 4 days incubation, cell viability was decreased with IC_{50} value was 1.00 ± 0.58 μM. CsA did not show acute cytotoxic effect against Jurkat T cells up to 1 μM after incubated for 1 and 4 days, IC_{50} values were 5.00 ± 0.07 and 3.00 ± 0.25 μM in 1 and 4 days, respectively. The results suggested that both FK506 and CsA contain anticancer activity.

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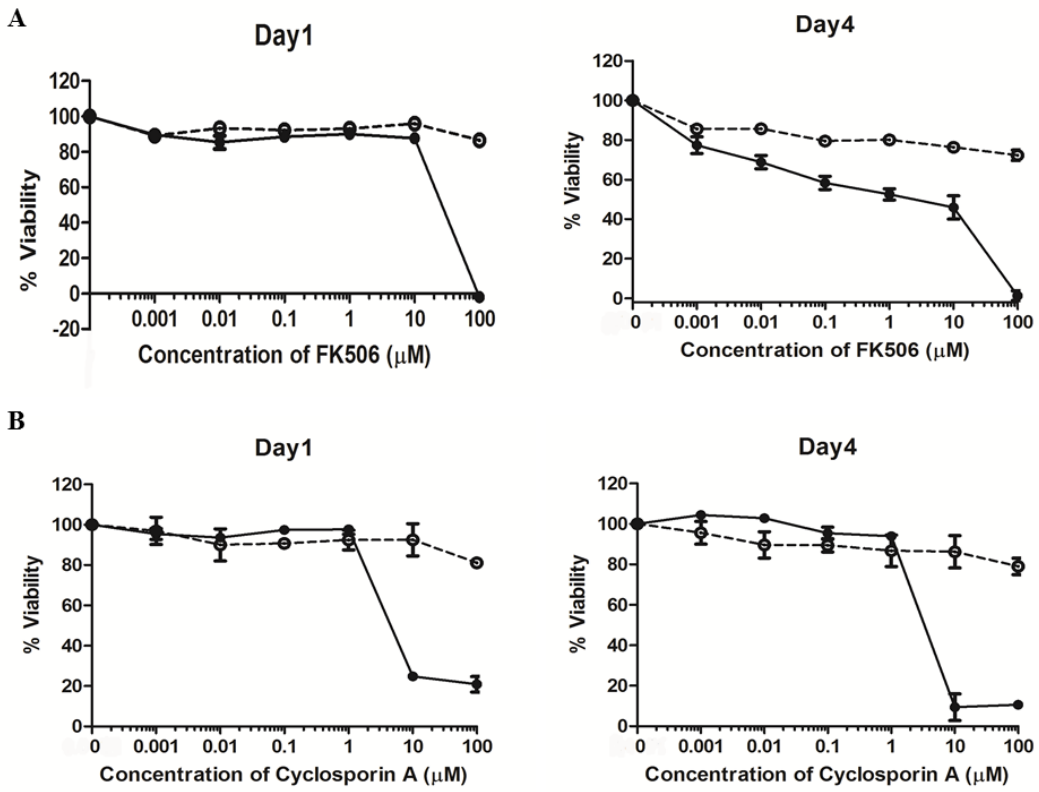

Figure 4.3 Cytotoxicity of FK506 and Cyclosporin A on Jurkat T cells assessed by MTT assays. Cells were treated with 0.001, 0.01, 0.1, 1, 10, 100 μM of FK506 (●) (A) and Cyclosporin A (●) (B) compared with DMSO control (○) incubated at 37°C in the atmosphere of 5% CO_2 for 1 day and 4 days, respectively. The data at each time point were mean ± SD from triplicate repeats.

4.5 Effect of CHA-01 on cell cycle progression of Jurkat T cells

To address whether CHA-01 affected cell cycle progression. Previous study, Suaum (2011) found that CHA-01 inhibited calcineurin function in similar manner to the known calcineurin inhibitors, FK506 and CsA. The effect of CHA-01 in comparison with FK506 and CsA on cell cycle progression of Jurkat T cells was observed. DNA content of Jurkat T cells treated for 24 h with or without CHA-01, FK506 or CsA was analyzed by flow cytometry.

The results showed that CHA-01 treatment caused an increase in the population of S phase cells in dose-dependent manner, from 17.45 ± 0.78% in the control treatment, to 21.00 ± 0.00%, 26.30 ± 0.42% and 27.85 ± 0.49% in the cells treated with 0.5, 1 and 2 µM CHA-01, respectively. Furthermore, an increase in the population of Sub G_1 phase cells, from 2.5 ± 0.07% in the control treatment to 3.7 ± 1.10%, 9.4 ± 1.56% and 12.1 ± 0.28% in the cells treated with 0.5, 1 and 2 µM CHA-01, respectively, was observed in Fig. 4.4A. FK506 treatment caused a subtle increase in the population of G_0/G_1 phase cells, from 45.2 ± 0.42% in the control treatment, to 49.5 ± 1.34%, 50.4 ± 0.85% and 51.6 ± 0.99% in the cells treated with 1, 2 and 4 µM FK506, respectively in Fig. 4.4B. The CsA treatment caused an increase in the population of subG_1 phase cells in dose-dependent manner, from 3.9 ± 1.11% in the control treatment, to 4.3 ± 0.13%, 10.3 ± 0.52% and 33.4 ± 1.84% in the cells treated with 3, 6 and 12 µM CsA, respectively in Fig. 4.4C.

The results suggested that CHA-01 treated Jurkat T cells were arrested at S phase of the cell cycle and led to apoptotic cell death as a significant increase in sub G1 population was noted. FK506, on the other hand, caused subtle G_0/G_1 phase cells accumulation. In case of CsA treament caused an enormous increase in the population of sub G_1 phase cells in dose-dependent manner. The results of CHA-01, FK506 and CsA treatments on cell cycle progression of Jurkat T cells were different. While FK506 and CsA treatment did not apparently affect cell cycle progression, however, CHA-01 treatment clearly demonstrated S phase cell accumulation. These differences suggested that CHA-01 might have other target beside calcineurin.

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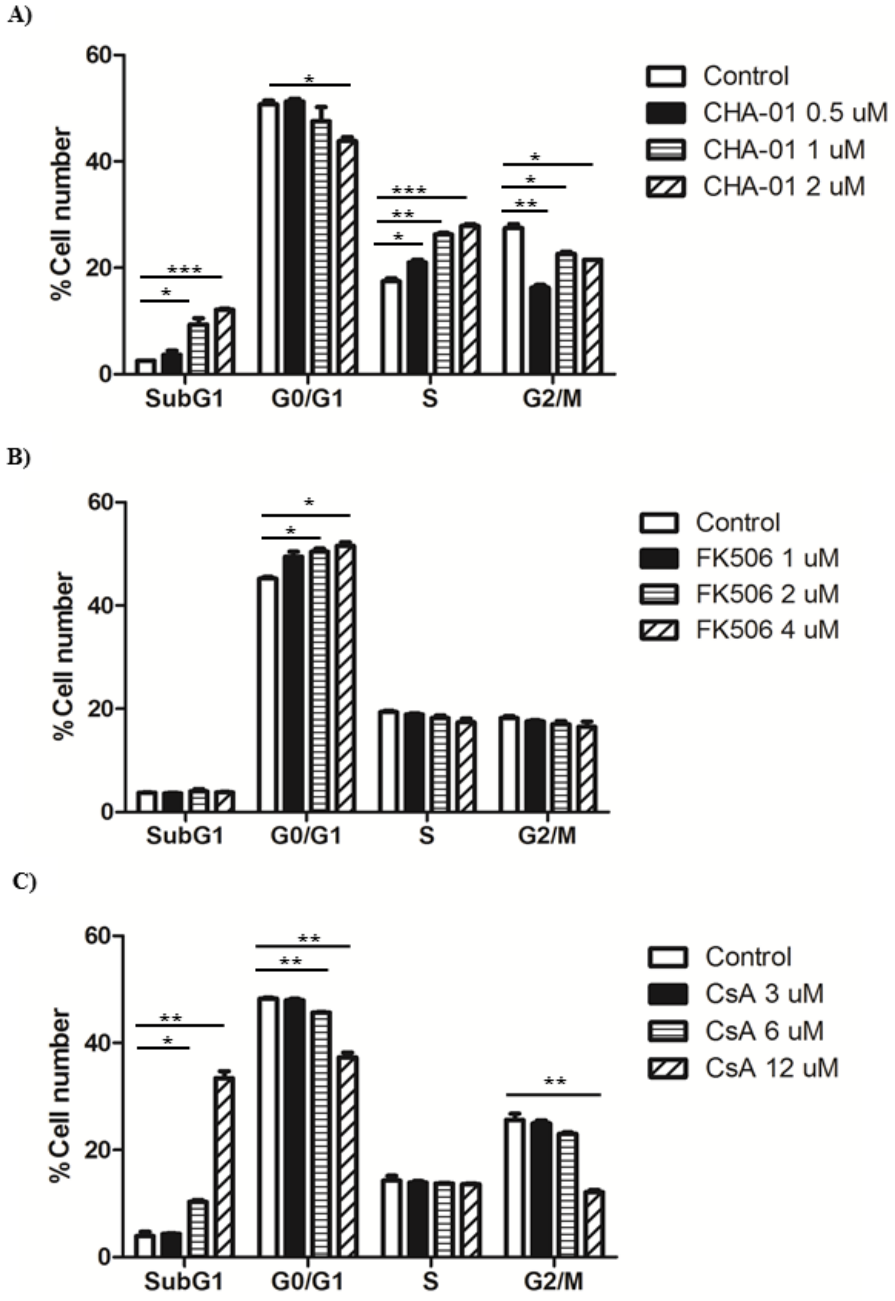

Figure 4.4 Effect of CHA-01, FK506 and CsA on cell cycle distribution in Jurkat T cells. The cells were incubated for 24 h at 37°C, 5% CO_2 in the absence (control, DMSO) or presence of CHA-01 (A), FK506 (B) and CsA (C), after which the DNA content was determined by flow cytometry. The data showed the values of mean ± SD from triplicate experiments. * Significant differences at *p*-value <0.05, ** *p*-value <0.01, *** *p*-value <0.001

4.6 CHA-01 induces apoptosis in Jurkat T cell.

Due to increased in sub G1 cells caused by 1 μM CHA-01 treatment in Jurkat T cells, a clearly I wondered whether CHA-01 could induce apoptosis in Jurkat T cells. Jurkat T cells were incubated with CHA-01 at concentration of 1 μM for 6, 12 and 24 h. After Annexin V-FITC and PI staining, flow cytometry analysis was performed.

The principle of annexin V-FITC is briefly summarized. Phosphatidylserine (PS) is normally confined in the inner membrane leaflet of viable cells. The translocation of PS to the exposed membrane surface is an early event in apoptosis. Another early membrane event that is characteristic of apoptosis is the eruption of cell surface blebs. It is mainly at the surface blebs that the PS is exposed (Casciola-Rosen et al. 1996, Darzynkiewicz et al. 1997). Binding of annexin V to apoptotic cells is a useful tool to quantitatively measure cells in the early and middle stages of apoptosis. Necrosis, or cell death by metabolic arrest and lysis, as distinguished from apoptosis, is defined by a general swelling of the whole cell and its constituent organelles. Because this process is caused by early permeabilization of the cell membrane (lysis), necrosis is easily detected *in vitro* by exposure to a DNA binding dye such as PI. The exclusion of such a hydrophilic dye indicates cell viability (Majno and Joris 1995).

Quadrant regions (Fig. 4.5A) were determined to divide cells into four different populations: Annexin V-/PI- cells (lower left quadrant) were considered as viable cells, Annexin V+/PI- cells (lower right quadrant) as early apoptotic, Annexin V+/PI+ cells (upper right quadrant) as late apoptotic, and Annexin V-/PI+ cells (upper left quadrant) as necrotic cells. The flow cytometry results revealed that 1μM CHA-01 induced early apoptosis in Jurkat T cells. Percentage of early apoptosis cells were

18.1% and 49.1% after exposure of CHA-01 for 12 and 24 h, respectively (Fig. 4.5E). While in controls, early apoptosis rate were 2.63% and 2.13% for 12 and 24 h, respectively (Fig. 4.5A, B). The percentage of viable cells was significantly decreased (81.4% and 50.4% for 12 and 24 h) (Fig. 4.5D), whereas the percentage of late apoptotic cells (0.56% and 0.59% for 12 and 24 h) and necrotic cells (0.042% and 0.019% for 12 and 24 h) was not significant (Fig. 4.5B, 4.5C). The results clearly indicated that CHA-01 could induce apoptosis.

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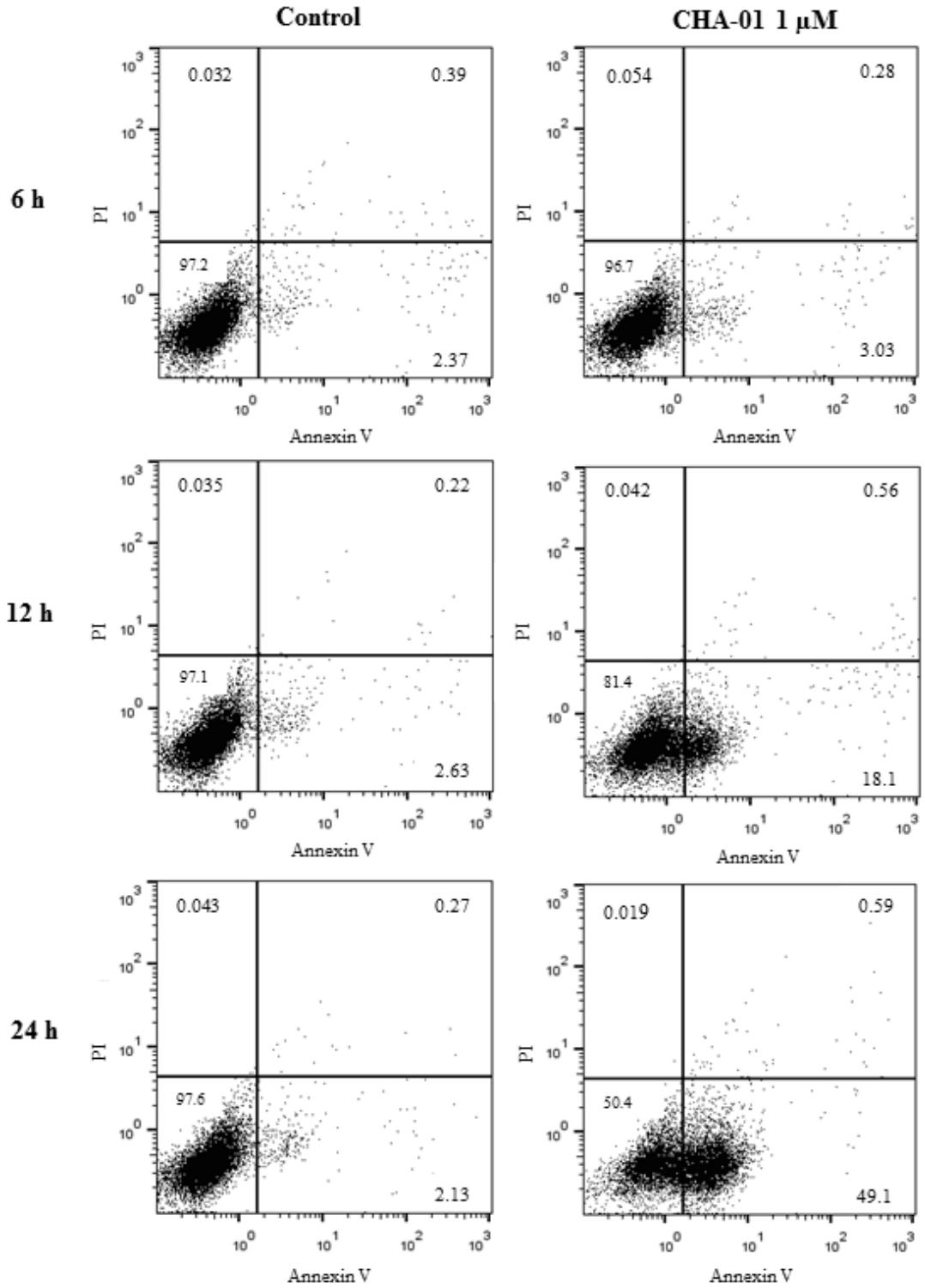
A)


Control
CHA-01 1 µM
6 h
12 h
24 h
PI
Annexin V
0.032
0.39
97.2
2.37
0.054
0.28
96.7
3.03
0.035
0.22
97.1
2.63
0.042
0.56
81.4
18.1
0.043
0.27
97.6
2.13
0.019
0.59
50.4
49.1

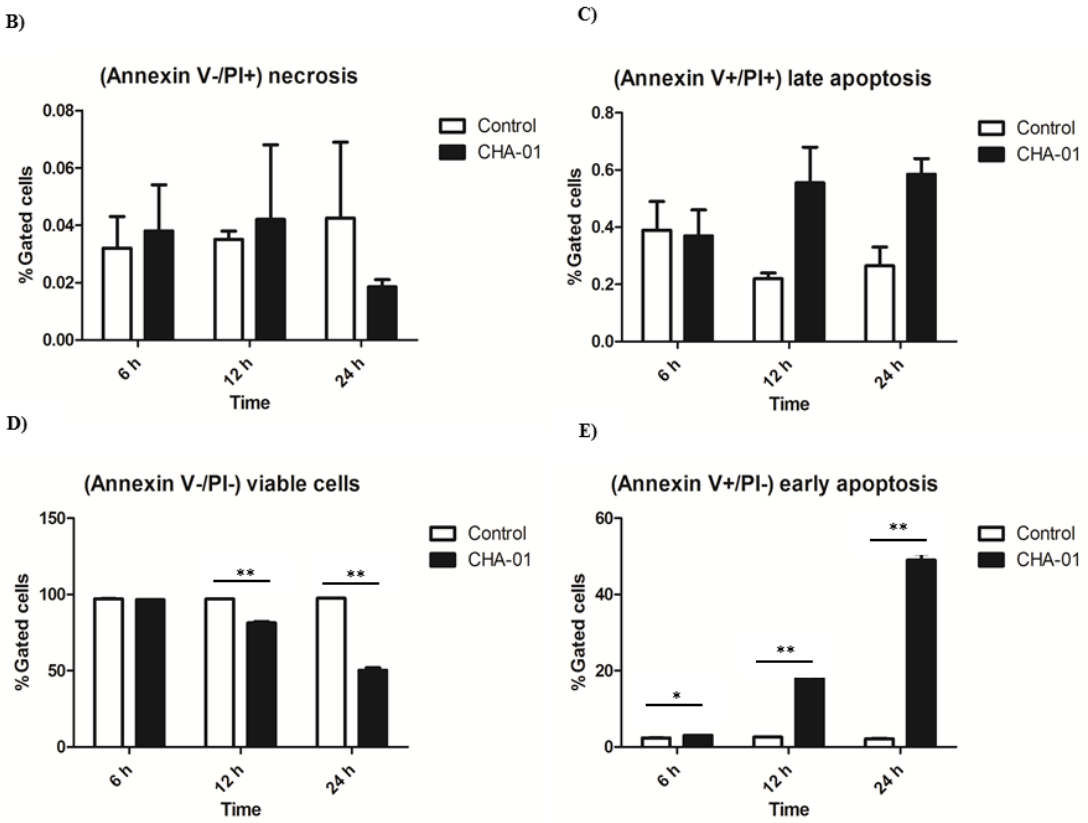


Figure 4.5 CHA-01 induced apoptosis in Jurkat T cell. The cells were incubated for 6, 12, 24 h in the absence (control, DMSO) or presence of 1 μM CHA-01 at 37°C in the atmosphere of 5% CO_2 (A) Original dot plots of the PI versus the Annexin V-FITC fluorescence intensities. Annexin V-FITC negative/PI negative region of the lower left quadrant are counted as viable cells. Annexin V-FITC positive/PI negative region of the lower right quadrant are counted as early apoptotic cells. Annexin V-FITC positive/PI positive region of the upper right quadrant are counted as late apoptosis cells. Annexin V-FITC negative/PI positive region of the upper left quadrant are counted as necrotic cells. Bar diagrams show the percentages of (B) necrotic, (C) late apoptotic, (D) viable and (E) early apoptotic cells. The data shown are representative of three independent experiments with the similar results. * p <0.05 and ** p <0.01 as compared with the control group.

4.7 DNA synthesis inhibition

CHA-01 treatment caused increase in the population of S phase cells. The accumulation of cells in S phase suggested that pathways concerning DNA synthesis might be inhibited by CHA-01. Thus, the effect of CHA-01 and other cytotoxic agents on DNA synthesis was investigated.

Bromodeoxyuridine (BrdU) incorporation assays used to detect DNA synthesis in vivo and in vitro. Bromodeoxyuridine (BrdU) is a thymidine analog.
The thymidine analog bromodeoxyuridine (BrdU) is incorporated into newly synthesized DNA in cells entering and progressing through the S (DNA synthesis) phase of the cell cycle (Gratzner and Leif 1981, Miltenburger et al. 1987). The incorporated BrdU is then stained with specific fluorescently labeled anti-BrdU antibodies, and the levels of cell-associated BrdU were measured using a flow cytometry. A dye that binds to total DNA, such as propidium iodide (PI), is often used in conjunction with immunofluorescent BrdU staining. This combination, two-color flow cytometric analysis permits the enumeration and characterization of cells that are actively synthesizing DNA (BrdU incorporation) relative to their phase in the cell cycle (ie, G_0/G_1, S, or G_2/M phases defined by PI staining intensities) (Dean et al. 1984, Lacombe et al. 1988).

Regions for the quantitative cell cycle analysis of populations that have been stained for incorporated BrdU and total DNA levels (Fig. 4.6)

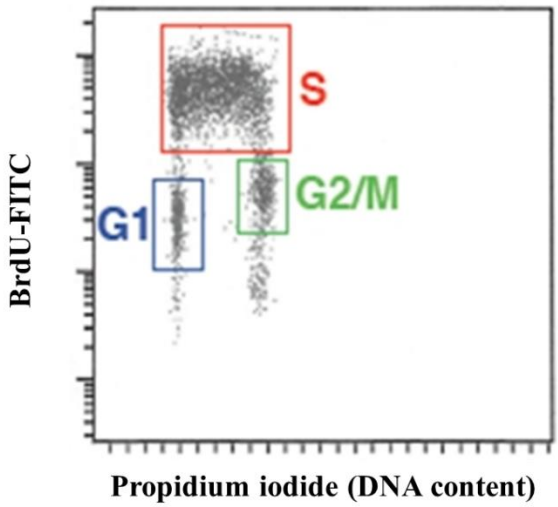


Figure 4.6 Typical bivariate plot showing DNA content (propidium iodide, X axis) and BrdU incorporation (FITC staining, Y axis). The G1, S and G2/M phase of the cell cycle can be better delineated (Ryba et al. 2011).

The accumulation of cells in S phase in CHA-01 treated Jurkat T cells suggested that pathways concerning DNA synthesis might be inhibited by CHA-01. Thus, the effect of CHA-01 on DNA synthesis was investigated. BrdU incorporation using an anti BrdU-FITC monoclonal antibody was measured by flow cytometry in CHA-01 treated Jurkat T cells.

Biparametric histograms of BrdU-FITC fluorescence versus PI fluorescence were shown in Fig. 4.7. Population of cells was found to accumulate in middle S phase. The amount of BrdU incorporation was decreased when treated with 1, 2, 4 or 8 µM of CHA-01 (with % S-phase cells 26.9, 24.9, 16.8, 15.6% vs that of control of 57.3%) for 24 and 48 h compared to those of controls (with the % of S-phase cells of 4.3, 4.3, 3.38, 2.54% vs that of control of 50.3%). The results demonstrated that accumulation of cells in S phase was the result of the DNA synthesis inhibition.

Other cytotoxic agents known to interfere with DNA synthesis were tested to compare their effects with that of CHA-01. Jurkat T cells were incubated for 24 h and 48 h with concentration at IC_{50} value of known drug, 10 nM camptothecin or 1 μM gemcitabine. It was found that only gemcitabine treatment revealed its effect on DNA synthesis that was somewhat similar to that of CHA-01 treatment after 48h incubation (Fig. 4.8). These results suggested that CHA-01 possesses a DNA synthesis inhibiting activity.

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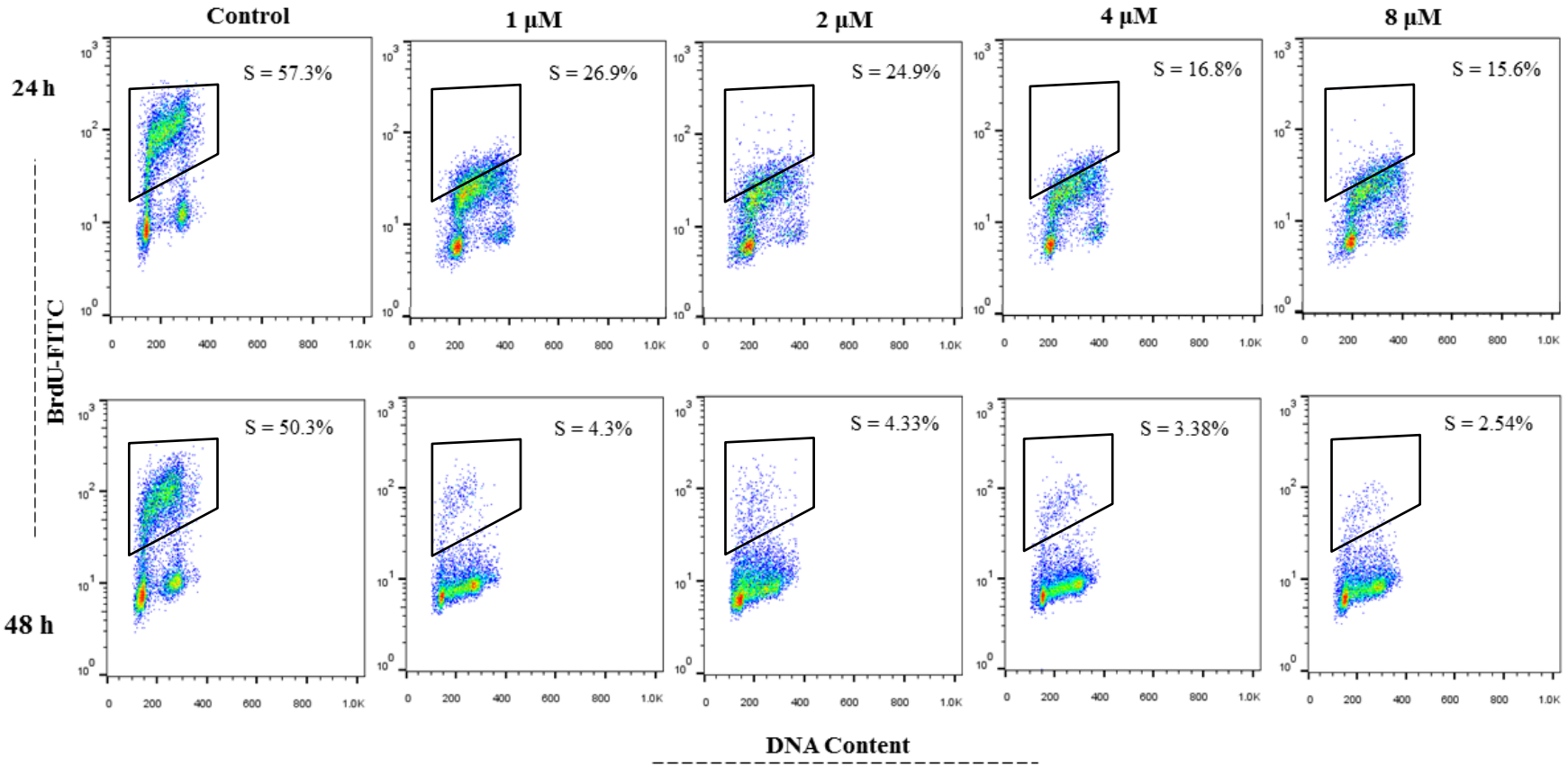

Figure 4.7 Effect of DNA synthesis inhibitors on BrdU incorporation. Jurkat T cells were treated for 24 h or 48 h at 37°C in the atmosphere of 5% CO_2 without or with 1, 2, 4 or 8 µM Clausmarin A and the cells were incubated for 1 h with BrdU and analyzed by flow cytometer. S: S phase cells.

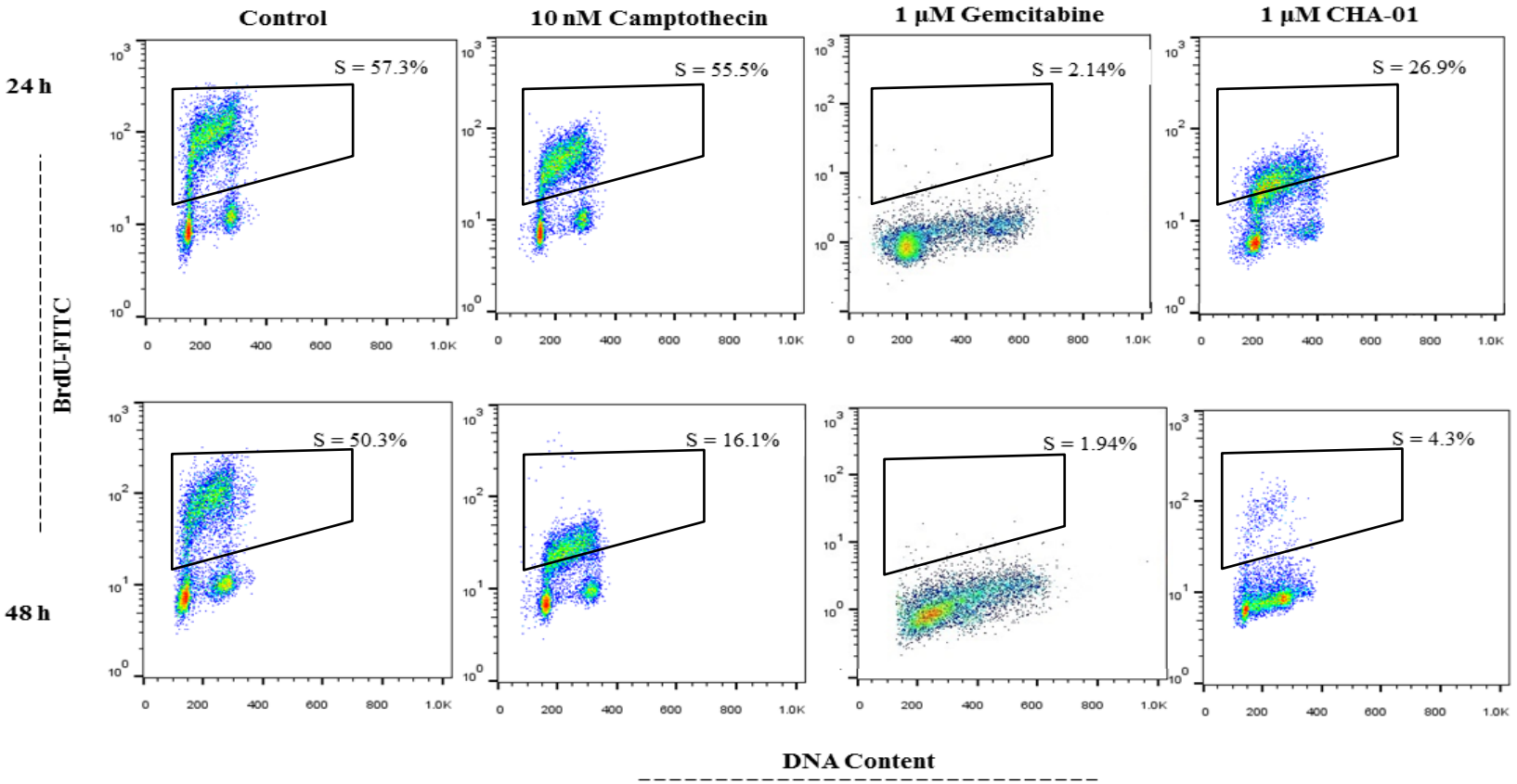

Figure 4.8 Effect of DNA synthesis inhibitors on BrdU incorporation. Jurkat T cells were treated for 24 h or 48 h at 37°C in the atmosphere of 5% CO_2 with 10 nM camptothecin, 1 µM gemcitabine and1 µM CHA-01 and incubated for 1 h with BrdU and analyzed by flow cytometry. S: S phase cells

4.8 Proteomic analysis of CHA-01 treated with Jurkat T cell

To search for proteins involving in anti-proliferation activity, S phase arrest, apoptosis induction and DNA synthesis inhibition in Jurkat T cells by CHA-01 treatment, the cellular proteome were investigated.

Proteins were extracted from Jurkat T cell treated with CHA-01. Then were isolated and separated by one-dimension gel electrophoresis (SDS-PAGE) as shown in Fig. 4.9. The protein expression patterns of Jurkat T cell after exposure to DMSO (control) and CHA-01 at 0, 15 and 60 min, respectively.

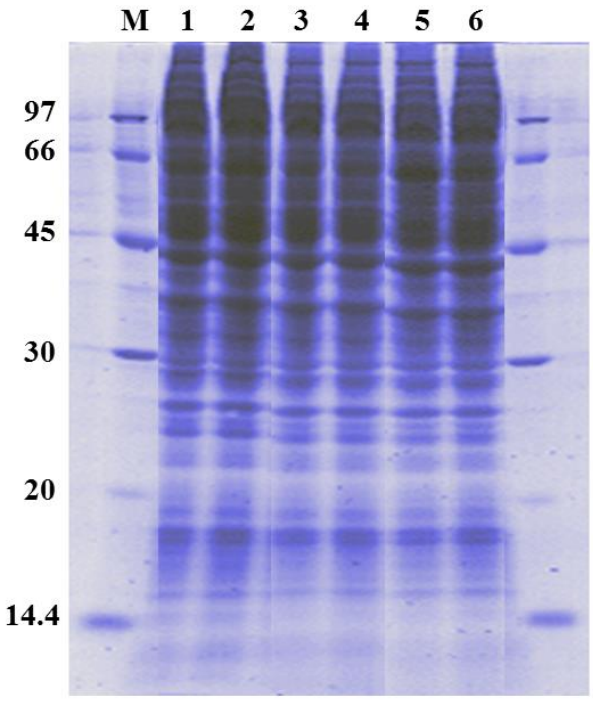

Figure 4.9 SDS-gel image of protein pattern between DMSO (control) compared to CHA-01 in Jurkat T cells at early time (0, 15 and 60 min). Lane 1, 3 and 5 showed the protein patterns of Jurkat T cells after exposure to DMSO for 0, 15 and 60 min, respectively. Lane 2, 4, and 6 showed the protein bands of CHA-01 treated Jurkat T cells at 0, 15 and 60 min, respectively.

After dividing the proteins in each samples according to molecular size, gel slice was subjected to in-gel tryptic digestion and peptides were analyzed by LC-MS/MS. The peptides in each samples was quantified by DeCyder MS Differential Analysis software and the differentially expressed peptides were submitted to database search using the Mascot program. A total of 861 proteins were identified. The biological processes of the proteins identified were assigned according to the KEGG (http://www.genome.jp/kegg/) and UniProt database (http://www.uniprot.org).

They were classified to play role in cell cycle (6, 0.70%), glycolysis (10, 1.17%), hypothetical protein (10, 1.17%), calcium regulation (5, 0.58%), transport (11, 1.28%), apoptosis (4, 0.47%), regulation (12, 1.40%), biosynthetic process (15, 1.75%), signaling (33, 3.85%), metabolic process (45, 5.24%), unnamed protein (155, 18.07%), others (439, 51.17%) and unknown function (113, 13.117%) as shown in Fig. 4.10.

Based on previous results, proteins playing role in cell cycle and apoptosis were chosen for Quantitative real-time RT-PCR shown in Fig. 4.11 and Table 4.2.

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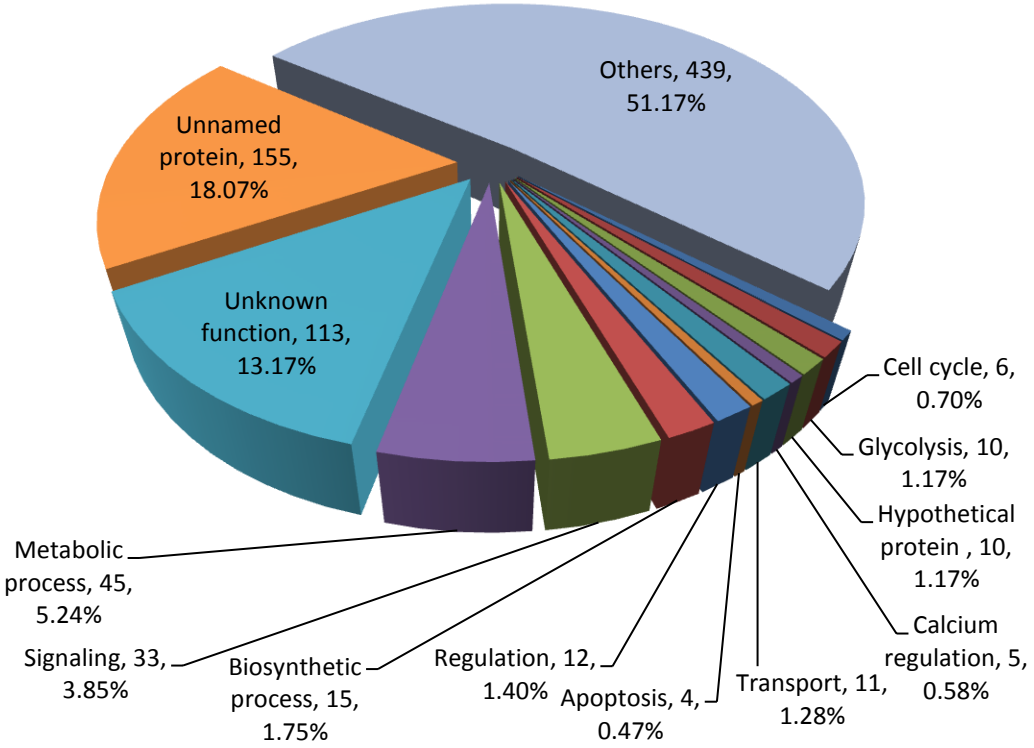

Figure 4.10 A pie chart showing the functional categories of differential expressed proteins found in Jurkat T cells treated with 1 μM CHA-01.

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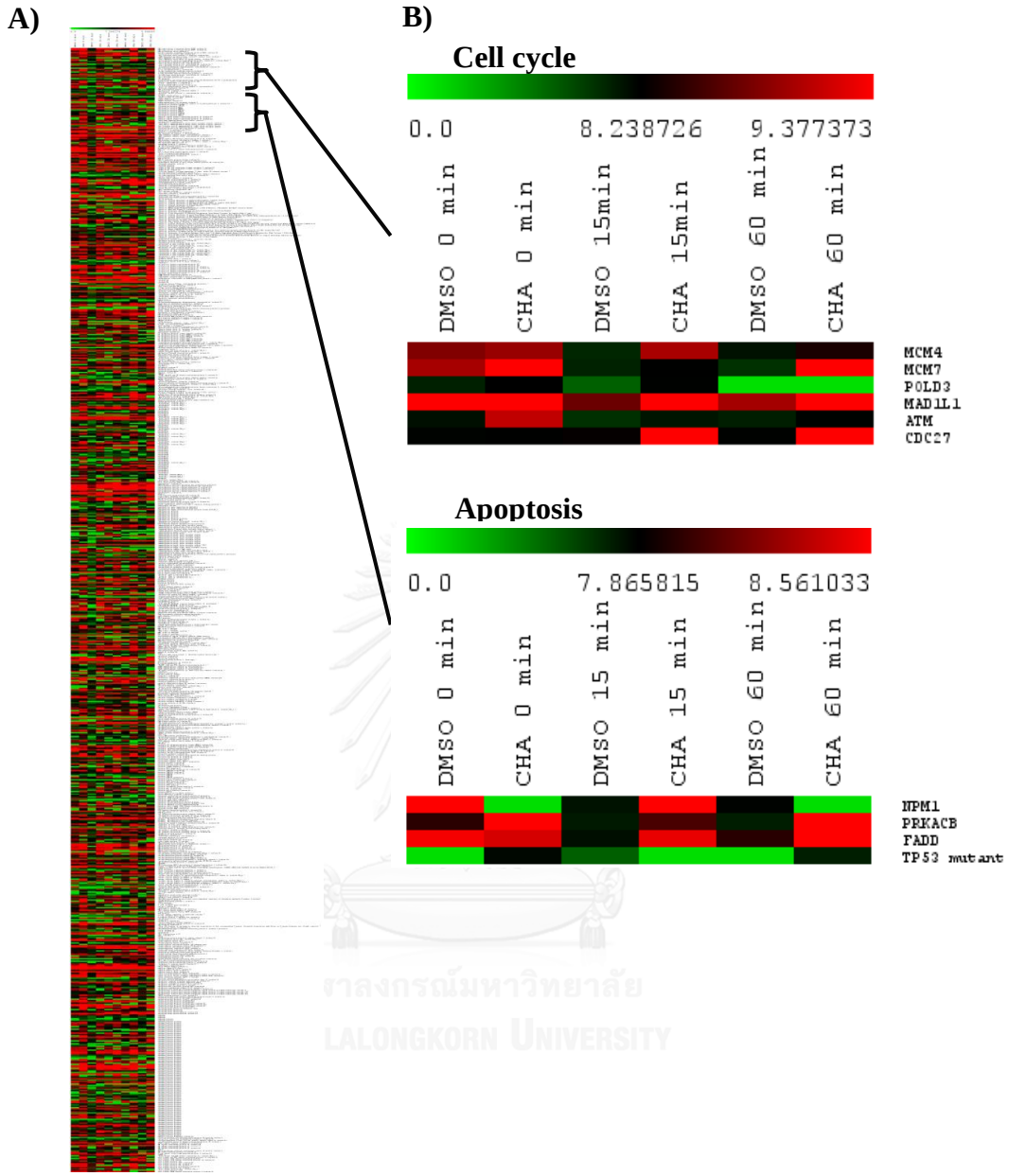

Figure 4.11 Heatmap of the quantitative differential expression of total proteins (861 proteins) among control (DMSO) and CHA-01 treated Jurkat T cells at 0, 15 and 60 min, respectively. Each row represents an individual protein and each column represents an individual sample (bar chart represents the signal intensity level as shown in corresponding color) (A). Function of proteins are involved in cell cycle and apoptosis were shown (B).

Table 4.2 Identification of expressed proteins found in cell cycle and apoptosis group at early time (0, 15 and 60 min)

Protein name	Accession no.	Peptide	ID score
Cell cycle			
DNA replication licensing factor MCM4 isoform X1 (MCM4)	gi|530388425	TTIENIQLPHTLLSR	23.03
DNA replication licensing factor MCM7 isoform X1 (MCM7)	gi|530385909	IAQPGDHVSVTGIFLPILR	39.22
DNA polymerase delta subunit 3 (POLD3)	gi|38492356	KGTAALGKANRQVSITGFFQR	10.55
mitotic spindle assembly checkpoint protein MAD1 isoform X4 (MAD1L1)	gi|578813662	SHLIQVEREK	10.58
serine-protein kinase ATM isoform X4 (ATM)	gi|578822211	SCYKVLIPHLVIR	18.74
Identification of expressed proteins found in cell cycle and apoptosis group at early time (0, 15 and 60 min)			12.09
Apoptosis			
cAMP-dependent protein kinase catalytic subunit beta isoform 3 (PRKACB)	gi|46909587	MGNAATAKKGSEVESVKEFLAK	13.79
tumor protein p53 mutant form (TP53)	gi|94442887	RPILTIITLEDSRPR	9.3
Fas (TNFRSF6)-associated via death domain (FADD)	gi|119595168	ADRAQVPMPR	14.09
nucleophosmin (nucleolar phosphoprotein B23, numatrin) (NPM1)	gi|119581848	VKLAADEDVMMMMILMMR	12.48

To validate the level of differential expressed proteins in Jurkat T cells during CHA-01 treatment, the transcriptional level of genes involving in cell proliferation activity, cell cycle at S phase, apoptosis induction and DNA synthesis were evaluated. Level of proteins and the corresponded mRNA in CHA-01 treated Jurkat T cells subtracted with those of control treatment (DMSO) were shown in figures 4.12 and 4.13.

From 861 total protein obtained, 5 candidate proteins suggested by KEGG and uniplot databases that involved in cell cycle and apoptosis were selected: MCM4, POLD3, NPM1, PRKACB and FADD. The candidate genes were further cloned and studied on mRNA expression levels.

Candidate proteins that involved in cell cycle were as follows: DNA replication licensing factor MCM4 isoform X1 (MCM4), a member of minichromosome maintenance protein family, involved in initiation of eukaryotic genome replication (Honeycutt et al. 2006). DNA polymerase delta subunit 3 (POLD3) plays a critical role in DNA replication, DNA repair and DNA damage response (Takahashi et al. 2015).

Candidate proteins that involved in apoptotic process were as follows. Nucleophosmin (nucleolar phosphoprotein B23, numatrin) (NPM1) have a role in the regulation of cell growth, proliferation and transformation (Feuerstein et al. 1988). NPM1 might have oncogenic and tumour-suppressing functions, depending on its dosage and level of expression (Grisendi et al. 2006). cAMP-dependent protein kinase catalytic subunit beta isoform 3 (PRKACB) is a member of the Ser/Thr protein kinase family and a key effector of the cAMP/PKA-induced signaling pathway that is involved in numerous cellular processes, including cell proliferation and apoptosis

(Skålhegg and Tasken 2000). Fas (TNFRSF6)-associated via death domain (FADD) is an apoptotic adaptor molecule that recruits caspase-8 or caspase-10 to the activated Fas (CD95) or TNFR-1 receptors. The resulting aggregate called the death-inducing signaling complex (DISC) (Walsh et al. 1998). All 5 candidate genes, *MCM4, POLD3, NPM1, PRKACB, and FADD*, were down-regulated within 15 min after CHA-01 treatment (Fig. 4.12A, B, C, D and E, respectively). At 60 min of treatment, MCM4, POLD3 and NPM1 showed decrease in the protein levels as compared to at 0 min (Fig. 4.13A, B and C). In case of *FADD*, the mRNA level tended to increase after 15 to 60 min of treatment (Fig. 4.12E) and its protein level remain higher than at 0 min (Fig. 4.13E). For *PRKACB* mRNA level at 60 min still remained lower than at 0 min (Fig. 4.12D), its protein level tended to rise up from 15 min to 60 min (Fig. 4.13D).

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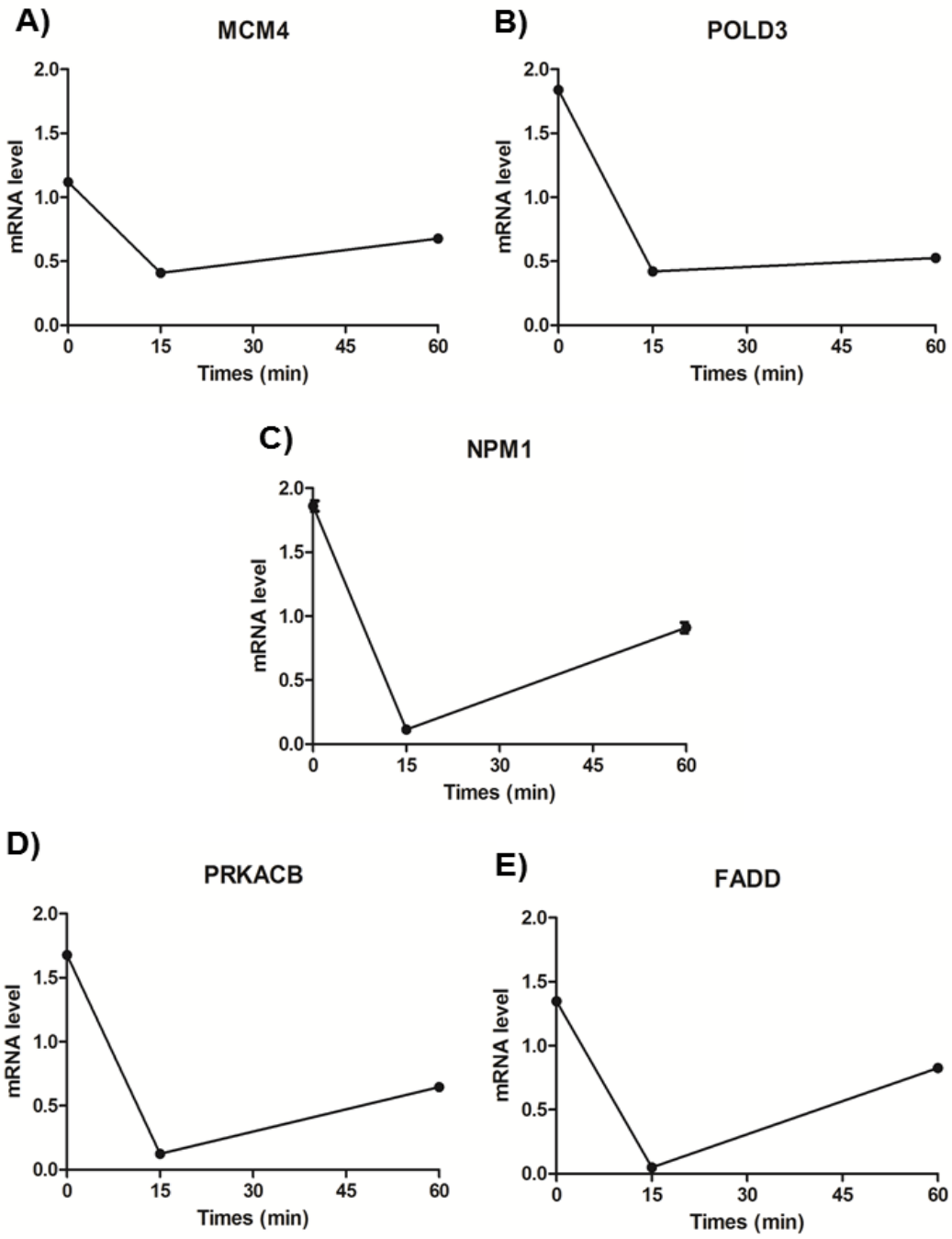

Figure 4.12 The relative expression levels of *MCM4* (A), *POLD3* (B), *NPM1* (C), *PRKACB* (D) and *FADD* (E) mRNA were determined by real time RT-PCR.

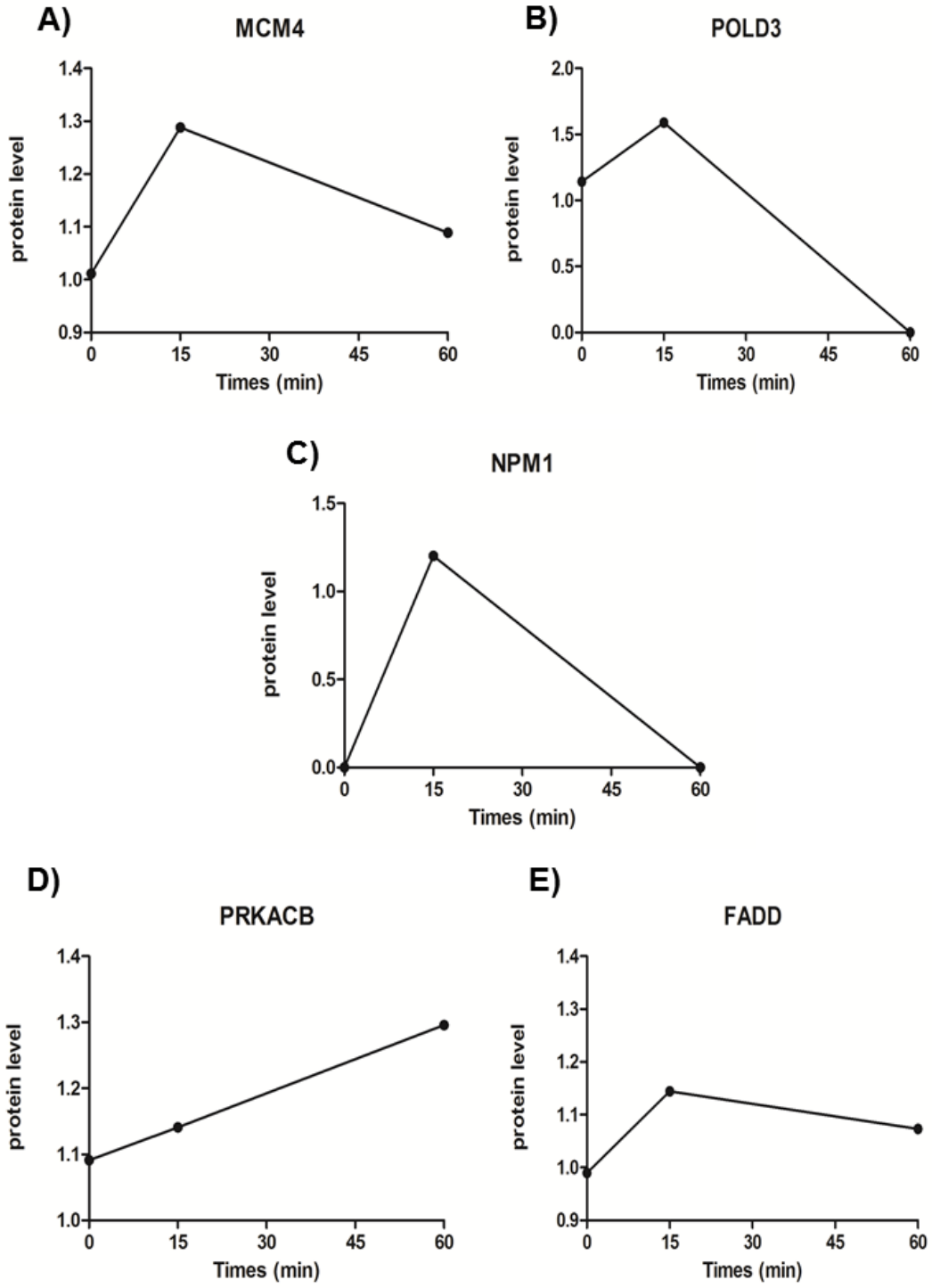

Figure 4.13 The relative expression levels of MCM4 (A), POLD3 (B), NPM1 (C), PRKACB (D) and FADD (E) protein were determined from peptide intensity data (after normalization).

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

Previously, coumarin compounds have been reported to inhibit cell growth in various human malignant cell lines (LNCaP, PC-3 (Prostate carcinoma), ACHN (Renal cell carcinoma), H727 (Malignant carcinoid), HS-Sultan (Myeloma (IgG)) cell lines) (Marshall et al. 1994), human lung carcinoma cells (Lopez-Gonzalez et al. 2004), human cervical cancer HeLa Cells (Chuang et al. 2007). As the anti-cancer activity of coumarin compound CHA-01 has not yet been reported, in this studies, I found that CHA-01, could inhibit cellular proliferation of Jurkat T cells, KATO-III and THP-1 in a dose- and a time dependent manner. CHA-01 did not showed acute cytotoxic effects against Jurkat T cell, KATO III when were treated with CHA-01 for 1 day (Fig. 4.1A, B, Left panel), however, it cause chronic cytotoxic effect against all 3 cell lines tested (Fig. 4.1, Right panel). THP-1 is rather sensitive to CHA-01 even at 1 day of treatment. Jurkat T cell line was chosen for further evaluation on effect of CHA-01 because it clearly showed the most sensitive to the effect of chronic cytotoxicity of CHA-01 treatment while no acute cytotoxic effect at rather high dose as up to 10 μM (Fig. 4.1A). Furthermore CHA-01 displayed no cytotoxic effect to the normal human white blood cells (up to 10 μM) (Fig. 4.2) and human red blood cells (up to 100 μM) (Table 4.1).

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CHA-01 caused S phase arrest in the treated Jurkat T cells (Fig. 4.4A). Some coumarin compounds were previously reported to cause S phase cell cycle arrest in many cancer cell lines. Xanthoxyletin, a coumarin compound, arrested the cell cycle of human gastric adenocarcinoma SGC-7901 cells at S phase (Rasul et al. 2011). Esculatin, a coumarin derivative blocked the cell cycle at S phase of SMMC-7721 cells (human hepatocellular carcinoma cell line) (Wang et al. 2015). However, no report on the detail molecular mechanism has been proposed.

Suaum (2011) found that CHA-01 is a calcineurin inhibitor acting rather similar to FK506 and CsA, the known calcineurin inhibitor in suppression of T cell proliferation. In this study, I found that the cell cycle profile of CHA-01 treated cells exhibited differently from those of FK506 or CsA treated cells (Fig. 4.4). I found that FK506 caused subtle G_0/G_1 phase of the cell cycle arrest. The results are in agreement with previous report by (Choi et al. 2008). I also found no change in cell cycle progression in CsA treated Jurkat cells except causing increase in Sub G1 population (Fig. 4.4C). Previous study also demonstrated that CsA-induced apoptosis and release of bioactive TGF-β1 in Jurkat T cells (Minguillon et al. 2005). By contrast to those found in FK506 and CsA, CHA-01 clearly caused cell cycle of Jurkat cells arrest at S phase and increased in the sub G1 population and as a result might lead to apoptosis (Fig. 4.4A). Previous studies have shown that coumarin compound have a potent growth inhibition in many cancer cell lines through induction of apoptosis (Lopez-Gonzalez et al. 2004, Murata et al. 2008, Rasul et al. 2011).

The accumulation of CHA-01 treated Jurkat cells in S phase suggested that pathways concerning DNA synthesis might be inhibited by CHA-01. BrdU incorporation was decreased in Jurkat cells when were treated with varying

concentration of CHA-01 (1, 2, 4, 8 μM) for 24 and 48 h. The BrdU incorporation was decreased as dosage of CHA-01 increased and also as time of exposure increased (Fig. 4.7). Other cytotoxic agents known to interfere with DNA synthesis were tested to compare their effects with those of CHA-01. Jurkat T cells were incubated for 24 h and 48 h with 10 nM camptothecin or 1 μM gemcitabine. By comparing the pattern of cells incorporated BrdU in S phase, the results revealed that CHA-01 treated Jurkat T cells showed rather similar pattern to those treated with gemcitabine, however in lesser extent while showed no similar pattern to those treated with camptothecin (Fig. 4.8) The results indicated that CHA-01 inhibited DNA synthesis.

To understand more on molecular mechanism of CHA-01 on Jurkat T cells that involved in anti-proliferation activity, S phase cell cycle arrest, apoptosis induction and DNA synthesis inhibition, proteomic analysis of CHA-01 treated Jurkat T cells was investigated. From total 861 proteins obtained, 5 candidate proteins having functions involved in cell cycle and apoptosis were selected for studying gene expression levels (Figs. 4.12 and 4.13). There were *MCM4, POLD3, NPM1, PRKACB* and *FADD*.

MCM4, POLD3, NPM1, PRKACB, and *FADD* mRNA levels, were down-regulated within 15 min after CHA-01 (Fig. 4.12) treatment while the MCM4, POLD3, NPM1 protein levels were down regulated at 60 min after CHA-01 treatment (Fig. 4.13). Among these, POLD3 protein level showed the most obvious down regulated. While FADD and PRKACB protein levels shown up regulated at 60 min compared to that at 0 min.

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The model for the coumarin, CHA-01, action mechanism was, therefore, proposed in Figure 5.1 When Jurkat T cells were treated with CHA-01, CHA-01 entered into the cells via its specific receptor. The signals might send to the nucleus causing down regulation of MCM4, POLD3 and NPM1, resulting in inhibition of cell cycle progression and S phase arrest, inhibition of DNA synthesis and inhibition of DNA repair and cell proliferation. On supporting the proposed model, previous studies found that depletion of MCM caused an S-phase arrest (Creve et al. 2007) and depletion of POLD3 led to inhibit growth of U2OS cells overexpressing cyclin E (Costantino et al. 2014)

Another signals after CHA-01 entering into the cells might activate PRKACB a protein kinase and as a result, up regulation of ERK, p38 and JNK signaling pathways and leading to apoptosis induction (Shen et al. 2007). The signals might also activate FADD for interaction with caspase-8 or 10 leading to an activation of apoptosis. Furthermore, caspase-8 is responsible for other protein and get integrated into the mitochondrial membrane and may alter mitochondrial membrane permeability, causing mitochondrial swelling and eventual rupture of the outer membrane, thus releasing intermembrane proteins into the cytosol and leading to an activation of apoptosis (Hengartner 2000).

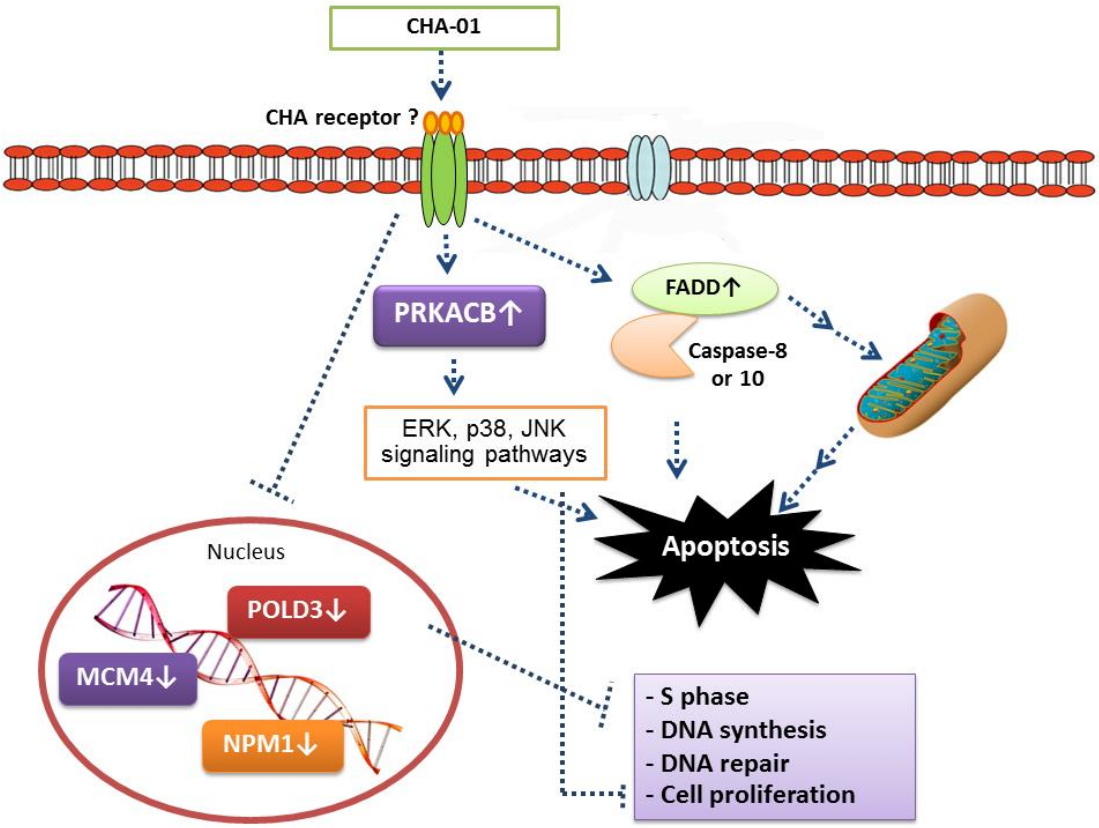

Figure 5.1 Proposed model on molecular mechanisms of CHA-01 in Jurkat T cells for 60 min of treatment.

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5.2 Conclusions

1. CHA-01 could inhibit cellular proliferation of Jurkat T cells, KATTO-III and THP-1 and Jurkat T cell line was the most sensitive to CHA-01 for chronic cytotoxicity.

2. CHA-01 displayed no cytotoxic effect to the normal human white blood cells (up to 10 μM) and human red blood cells (up to 100 μM).

3. CHA-01 inhibited the proliferation of Jurkat T cells by inhibition of DNA synthesis and hence arresting cell cycle progression at S phase.

4. The 5 protein candidates that involved in cell cycle and apoptosis were selected: MCM4, POLD3, NPM1, PRKACB and FADD.

5. The protein levels of the candidates were not quite correlated with their mRNA expression levels.

6. The model for molecular mechanism of CHA-01 activity in Jurkat T cells for 60 min of treatment was proposed in Figure 5.1.

5.3 Futher Study

Study on the effect of CHA-01 treatment in Jurkat T cells in later time response (*e.g.* for 12 and 24 h).

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APPENDIX

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APPENDIX A

The media preparation

RPMI-1640 Complete medid 100 ml

RPMI-1640 with L-Glutamine	90	ml
Fetal bovine serum	10	ml
Penicillin G (10^6 U/ml)	10	µl
Streptomycin (500 mg/ml)	50	µl
2-Mercaptoethanol	1	ml

RPMI freezing media

RPMI complete media	9	ml
DMSO	1	ml

FBS inactivation

FBS must be inactivated at 56˚C for 30 min using water bath before use.

Luria-Bertani broth (LB broth)

Yeast extract	0.5	g
Tryptone	1	g
NaCl	1	g
Distilled water	100	ml

Autoclave at 121˚C for 15 min

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Luria-Bertani agar (LA agar) containing ampicillin, IPTG and X-gal

Yeast extract	0.5	g
Tryptone	1	g
NaCl	1	g
Distilled water	100	ml

Autoclave at 121˚C for 15 min. Pour sterile warm LB agar into a Petri dish. Dry opened LB plates at room temperature under UV light for about 30 min. Add 100 µl of ampicillin (100 mg/ml), 40 µl of X-Gal solution (20 mg/ml) and 40 µl of 100 mM IPTG solution. Spread evenly on the plate with a sterile spreader.

SOC medium

Yeast extract	0.5	g
Tryptone	2	g
1 M NaCl	1	ml
1 M KCl	0.25	ml
Distilled water	97	ml

Autoclave at 121˚C for 15 min. Add 1 ml of filter sterilized 2 M Mg^{2+} and 2 M glucose solution. Adjust the volume to 100 ml with sterile distilled water.

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APPENDIX B
The chemical preparation

Pencillin G and Streptomycin solution

Pencillin G and Streptomycin were prepared at final concentration 10^6 U/ml and 500 mg/ml in sterile deionized water, respectively. The solution were sterilized by using 0.45 μM filter and kept in aliquots at -20˚C.

MTT solution (5mg/ml)

MTT	50	mg
ddH_2O	10	ml

Dissolved MTT in ddH_2O and filter by syringe filter pore size 0.45 μM. Aliquot 1 ml in microcentrifuge tube and store at 4°C.

0.04 N HCl in isopropanaol

Added HCl 0.331 ml in isopropanol 80 ml and adjust volume to 100 ml by isopropanol.

RNase A solution (10mg/ml)

Dissolved RNase 10 mg in ddH_2O 1 ml and store at 4°C.

Ethanol 70%

Ethanol 99%	700	ml
ddH_2O	300	ml

10X Phosphate buffer saline (PBS, Ca2+, Mg2+ free) pH 7.4

NaCl	80	g
KCl	2	g
Na_2HPO_4	14.4	g
KH_2PO_4	2.4	g

Dissolved in ddH_2O up to 1 L and adjusted pH up to 7.4 with 1N HCl or 1 N NaOH and autoclaved at 121°C, 15 psi for 15 min.

RIPA buffer

1 M Tris-HCl pH 7.4	0.05	ml
0.5 M NaCl	0.3	ml
20% NP-40	0.05	ml
10% Sodium deoxycholate	0.05	ml
20% SDS	0.005	ml
Phosphatase inhibitor	0.01	ml
Protease inhibitor (100X)	0.01	ml
ddH_2O	525	ml

10% SDS

Dissolved sodium dodecyl sulfate 10 g in distilled water up to 100 mL and autoclaved at 121°C, 15 psi for 20 min.

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5% SDS

Dissolved sodium dodecyl sulfate 2.5 g in distilled water up to 50 mL and autoclaved at 121°C, 15 psi for 20 min.

10% Ammonium persulfate (APS)

Dissolved Ammonium persulfate 0.5 g in ddH_2O 5 ml and kept at 4°C.

0.5 M Tris pH 6.8 solution

Dissolved trisma base 6.1 g in ddH_2O 80 ml. Adjusted pH up to 6.8 with 1N HCl or 1 N NaOH and adjust volume to 100 ml. Autoclaved at 121°C, 15 psi for 15 min.

1.5 M Tris pH 8.8 solution

Dissolved trisma base 136.28 g in ddH_2O 400 ml. Adjusted pH up to 8.8 with 1N HCl or 1 N NaOH and adjust volume to 500 ml. Autoclaved at 121°C, 15 psi for 15 min.

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5% Stacking gel

ddH_2O	1828.3	µl
40% Acrylamide	375	µl
0.5 M Tris pH 6.8	742	µl
10% SDS	90	µl
10% APS	0.05	µl
TEMED	23	µl

12.5% Separating gel

ddH_2O	4.1967	ml
40% Acrylamide	3.125	ml
1.5 M Tris pH 8.8	2.5	ml
10% SDS	0.125	ml
10% APS	0.05	ml
TEMED	0.0033	ml

Lowry protein assay solution

Solution A 10 ml

CTC	1.25	ml
20% Na_2CO_3	1.25	ml
0.8 N NaOH	2.5	ml
5% SDS	5.0	ml

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Solution B 3 ml

Folin-Ciocalteu phenol reagent	0.5	ml
ddH_2O	2.5	ml

CTC stock solution

0.2% $CuSO_4$	0.2	g
0.4% Tatalic acid	0.4	g
ddH_2O	100	ml

0.8 N NaOH

NaOH	1.6	g
ddH_2O	50	ml

20% Na_2CO_3

Na_2CO_3	10	g
ddH_2O	50	ml

2X loading buffer

10% SDS	4	ml
Glycerol 87%	2.29	ml
1.0 M Tris pH 6.8	1	ml
ddH_2O	2.71	ml
Bromphenol blue	0.001	g

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5X SDS loading sample buffer

250 mM TrisHCl pH 6.8	12.5	ml
10% SDS	10	g
30% Glycerol	30	ml
5% Beta-mercapitalethanol	5	ml
0.02% Bromophenol blue	52	ml

Coomassie Blue Staining SDS-PAGE R250

Staining solution 1 liter

Coomassie Brilliant Blue R250	2.5	g
Methanol	500	ml
Glacial acetic acid	100	ml
Ultrapure water	400	ml

Destaining solution 1 liter

Ethanol	165	ml
Glacial acetic acid	50	ml
Ultrapure water	785	ml

DEPC Water

Added 0.01 ml of diethylpyrocarbonate (DEPC) in ddH_2O and mix thoroughly. Let the DEPC-mixed water incubate for overnight at room temperature. Autoclaved at 121°C, 15 psi for 15 min.

70% Ethanol in DEPC water

Absolute ethanol	70	ml
DEPC water	30	ml

2% Agarose gel

1X TAE buffer	20	ml
Agarose gel	0.4	g

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Ethidiumbromide solution

Dissolved ethidiumbromide in 1X TAE buffer at final concentratiom 10 µg/ml and store in dark container or wrap container in aluminum foil.

50X TAE pH 8.0

Trisma base	242	g
Glacial acetic acid	57.1	ml

Dissolved Trisma base and acetic acid in 0.5 M pH 8.0 100 ml and adjust to 1000 ml by ddH_2O. Autoclaved at 121°C, 15 psi for 15 min.

Ampicillin 100 mg/ml

Weigh out 5 g of ampicillin sodium salt and dissolve in 50 ml of double distilled water in a 50 ml centrifuge tube. Filter sterilize through a 0.22 □m filter, using a 60 ml syringe, into a sterile 50 ml centrifuge tube. Aliquot 1 ml/tube into 1.5 ml eppendorf tubes and freeze at -20°C.

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1 M IPTG (isopropyl-beta-D-thiogalactopyranoside)

Dissolve 2.38 g of IPTG in 8 mL of distilled water. Bring to a final volume of 10 ml with distilled water. Filter sterilize with a 0.22 µm syringe filter. Store in 1ml aliquots at -20°C.

X-gal (5-Bromo-4-Chloro-3-Indolyl-ß-D-Galactopyranoside) 20 mg/ml

Dissolve 100 mg of X-Gal into 5mL of dimethyl sulfoxide (DMSO). Divide into 1ml aliquots. Store in dark by wraping the solution aliquots in aluminum foil. Store at -20°C. X-gal solutions do not need to be sterilized.

50 mM Ammonium bicarbonate (NH_4HCO_3)

Dissolved ammonium bicarbonate 3.953 g in distilled water and adjust volume to 40 mL

10 mM Dithiothreitol (DTT)

Dissolved dithiothreitol 1.54 g in distilled water and adjust volume to 40 mL

100 mM Iodoacetamide (IAA)

Dissolved iodoacetamide 18.5 g in distilled water and adjust volume to 20 mL

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30% Acetonitrile (ACN)

Added 12 ml of acetonitrile in ddH_2O and mix thoroughly.

50% Acetonitrile in 0.1% Formic acid

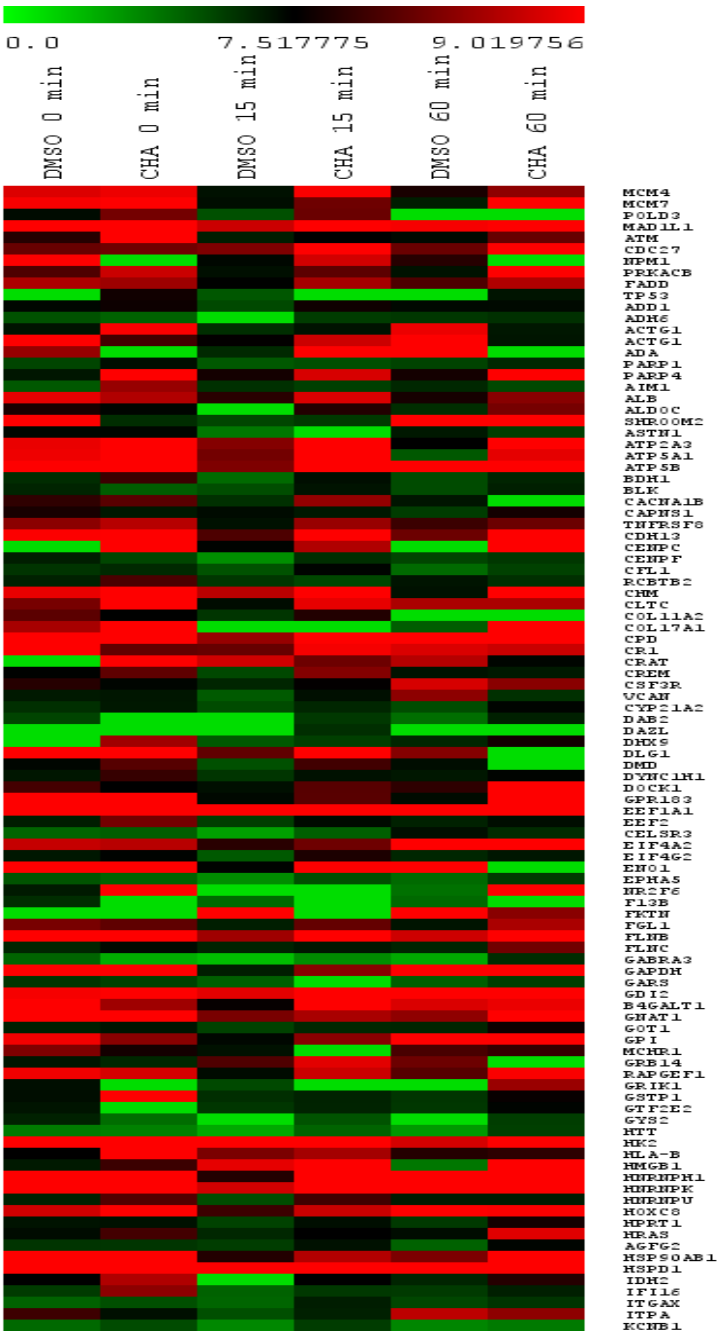
Added 100% acetonitrile in 100% of Formic acid 40 ml

APPENDIX C

Heatmap

1. Heatmap of the quantitative differential expression of total proteins (861 proteins) among control (DMSO) and CHA-01 treated Jurkat T cells at 0, 15 and 60 min

A)



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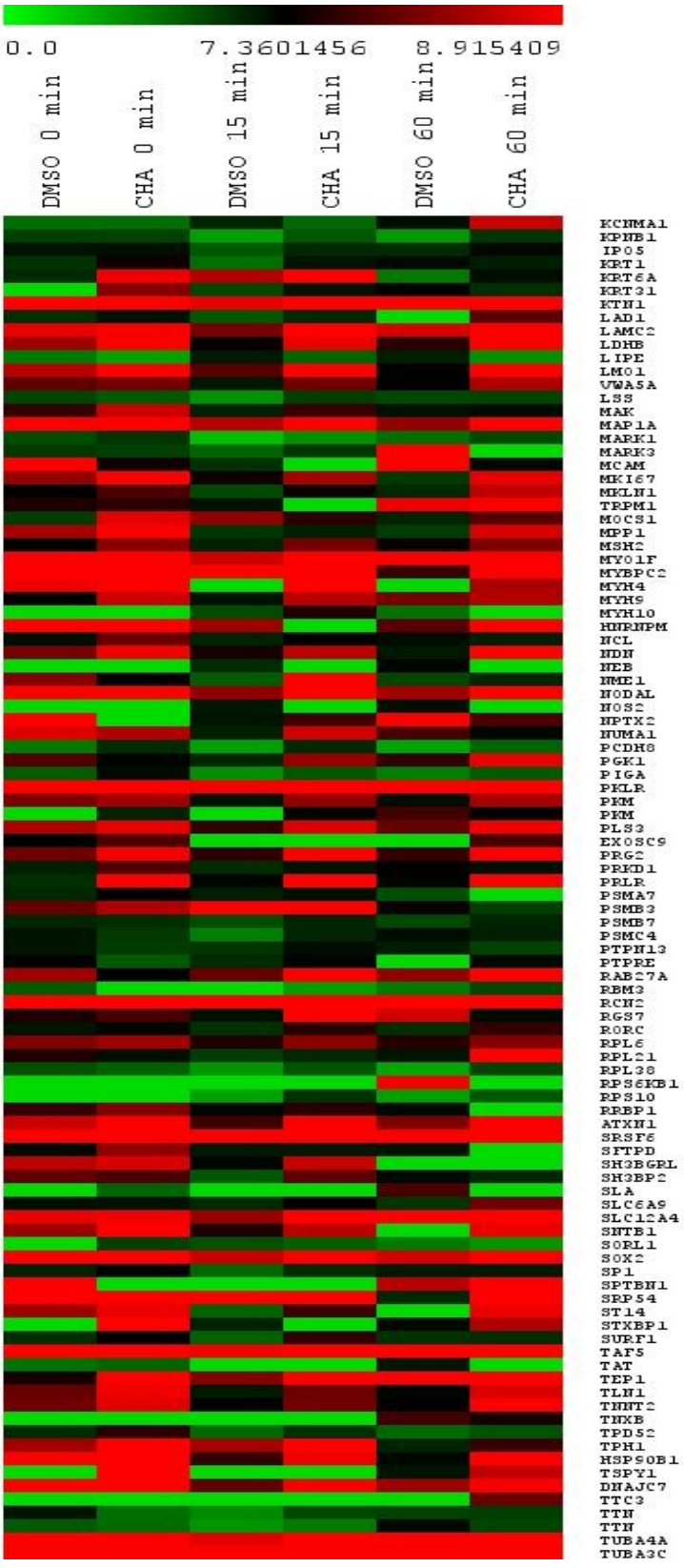
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8.915409
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CHA 0 min
DMSO 15 min
CHA 15 min
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CHA 60 min
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IP05
KRT1
KRT6A
KRT31
KTN1
LAD1
LAMC2
LDHB
LIPE
LMO1
VWASA
LSS
MAK
MAP1A
MARK1
MARK3
MCAM
MKI67
MKLN1
TRPM1
MOCS1
MPP1
MSH2
MY01F
MYBPC2
MYH4
MYH8
MYH10
HNRNPM
NCL
NDN
NEB
NME1
NODAL
NOS2
NPTX2
NUMA1
PCDH8
PGK1
PIGA
PKLR
PKM
PKM
PLS3
EXOSC9
PRG2
PRKD1
PRLR
PSMA7
PSMB3
PSMB7
PSMC4
PTPN13
PTPRE
RAB27A
RBM3
RCN2
RGS7
RORC
RPL6
RPL21
RPL38
RPS6KB1
RPS10
RRBP1
ATXN1
SRSF6
SFTPD
SH3BGRL
SH3BP2
SLA
SLC6A9
SLC12A4
SNTB1
SORL1
SOX2
SP1
SPTBN1
SRP54
ST14
STXBP1
SURF1
TAF5
TAT
TEP1
TLN1
THNT2
THXB
TPD52
TPH1
HSPS0B1
TSPY1
DNAJC7
TTC3
TTN
TTN
TUBA4A
TUBA3C

C)


0.0
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8.618462
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CHA 0 min
DMSO 15 min
CHA 15 min
DMSO 60 min
CHA 60 min
TXNRD1
TYK2
UBA52
UBA1
SUMO1
USP1
KDM6A
UTY
UTY
UTY
EZR
VIL2
VIPR1
C0R02A
XRCC5
RNF112
PRRC2A
DDX39B
TFEB
AKAP1
BAP1
D0C2A
CILP
PDXK
FAM193A
DNAH17
TNKS
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ADAM9
MPDZ
IQGAP1
SLC5A6
CPNE3
CACNA1I
HERC1
BSN
MBD2
RHBDL1
USP10
ARHGEF1
CHST3
ATG5
TBX4
MPDU1
CIR1
RBM39
NC0R2
NC0R2
ABCG1
CELSR1
MDC1
FAM53B
PPP6R2
KIAA0100
BCLAF1
DLGAP5
TRIM14
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FEM1B
ACTR1A
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RBM14
ZER1
T0MM40
UBE2E3
CCT7
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PAICS
KDM5B
STARD10
RUNDC3A
EDAR
EHMT2
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TRI0BP
DNAJB4
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NUDT6
ADAMTS6
CEP250
WBP4
PR0SC
TREX2

D)


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DMSO 60 min
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ABLIM3
SBNO2
AKR7A3
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RIMS1
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USP33
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EPB41L3
MAST2
GANAB
PMPCA
RRP12
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SLC9A8
TRIM2
SYNE1
UBR4
CRB1
GPR161
CBX5
TRAM1
SKIV2L2
GTPBP4
CCNDBP1
CARHSP1
AMACR
OSBP2
EML2
ZNF318
M0B4
POLR1A
C2CD2
DNAH1
SPATS2L
L3MBTL1
PHF19
EHF
CYFIP2
DNAJC2
VPS41
M0RC1
DISC1
R3HCC1L
RBMX
ZNF638
ACADS
RGCC
MRPL13
ANKRD11
BAZ2B
ST6GALNAC6
ERVW-1
ZBTB21
CD207
DUOX2
MYEF2
SLC35C2
MRPS16
KLHL5
CRIM1
KCNK9
FAM198B
PHF21A
CRNKL1
UBR5
C0MMD10
POLK
ZCCHC17
ZCCHC17
SLC25A39
NUP54
RIPK4
RBM27
ATAD2B
XRN1
CPVL
ANKRD16
SDK2
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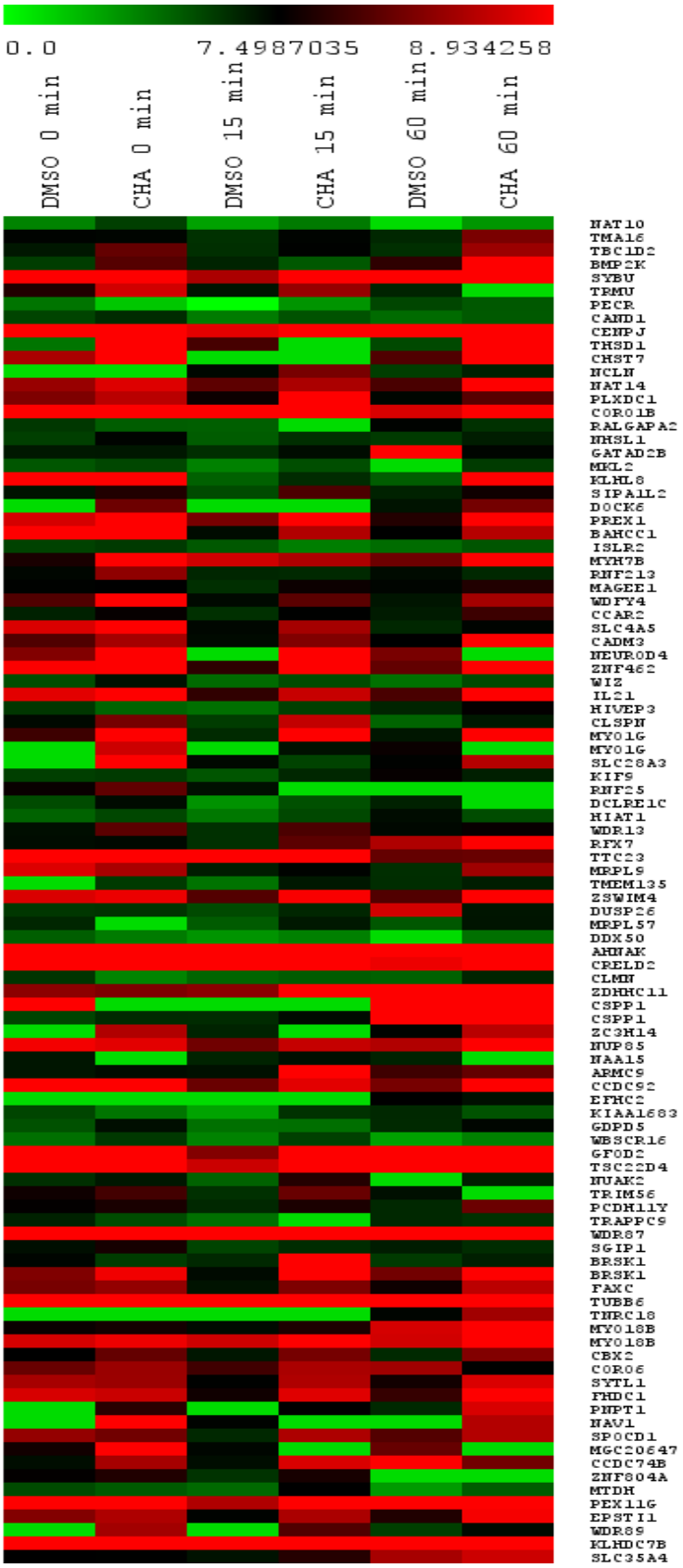
DEF8
CNTLN
FAM120C
C17orf80
SDAD1
LGI2
EXD2

E)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DMSO 15 min
CHA 15 min
DMSO 60 min
CHA 60 min
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TRMU
PECR
CAND1
CENPJ
THSD1
CHST7
NCLN
NAT14
PLXDC1
C0R01B
RALGAPA2
NHSL1
GATAD2B
MKL2
KLHL8
SIPA1L2
D0CK6
PREX1
BAHCC1
ISLR2
MYH7B
RNF213
MAGEE1
WDFY4
CCAR2
SLC4A5
CADM3
NEUR0D4
ZNF462
WIZ
IL21
HIVEP3
CLSPN
MY01G
MY01G
SLC28A3
KIF9
RNF25
DCLRE1C
HIAT1
WDR13
RFX7
TTC23
MRPL9
TMEM135
ZSWIM4
DUSP26
MRPL57
DDX50
AHNAK
CRELD2
CLMN
ZDHHC11
CSPP1
CSPP1
ZC3H14
NUP85
NAA15
ARMC9
CCDC92
EFHC2
KIAA1683
GDPD5
WBSCR16
GF0D2
TSC22D4
NUAK2
TRIM56
PCDH11Y
TRAPPC9
WDR87
SGIP1
BRSK1
BRSK1
FAXC
TUBB6
TNRC18
MY018B
MY018B
CBX2
C0R06
SYTL1
FHDC1
PNPT1
NAV1
SP0CD1
MGC20647
CCDC74B
ZNF804A
MTDH
PEX11G
EPSTI1
WDR89
KLHDC7B
SLC35A4

F)


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8.58163
DMSO 0 min
CHA 0 min
DMSO 15 min
CHA 15 min
DMSO 60 min
CHA 60 min
MBD6
FHAD1
OSBPL1A
SLC26A9
HSPA12B
PIK3AP1
CALHM3
NXPE1
FGD4
CSNK1A1L
NTAN1
C17orf64
SPATA32
NLRP8
C19orf47
ATXN7L2
EDARADD
SGSM1
UBR3
ENPP6
MROH2B
SV0PL
FAM92A1
LETM2
C20orf96
CTCFL
TRIM69
ABHD16B
SPTY2D1
AMDHD1
LYPD4
HIPK4
ATP8B3
ZNF555
KNCN
MEI1
FAM71B
SLFN5
DENND1B
C1orf177
PARP15
GPR125
LCA5
C0MMD6
ANKRD24
ZFC3H1
ZNF385C
DNAH12
CMYA5
TUBB
HNRNPA3
JMJD1C
NT5DC1
TBC1D32
ZBTB12
PRR15
FAM83B
LC0RL
PR0SER2
ANKRD31
HMCN2
EML3
MY01H
CCDC57
TTLL6
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HTR3E
PPP1R42
C9orf142
KLHL10
P0TED
TMEM225
FAM19A2
PRR30
WDR86
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RGSL1
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GBP7
FLG2
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ARHGEF37
IYD
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ZNF793
KN0P1
F0XL2NB
DDI1
UNC13C
CCDC73
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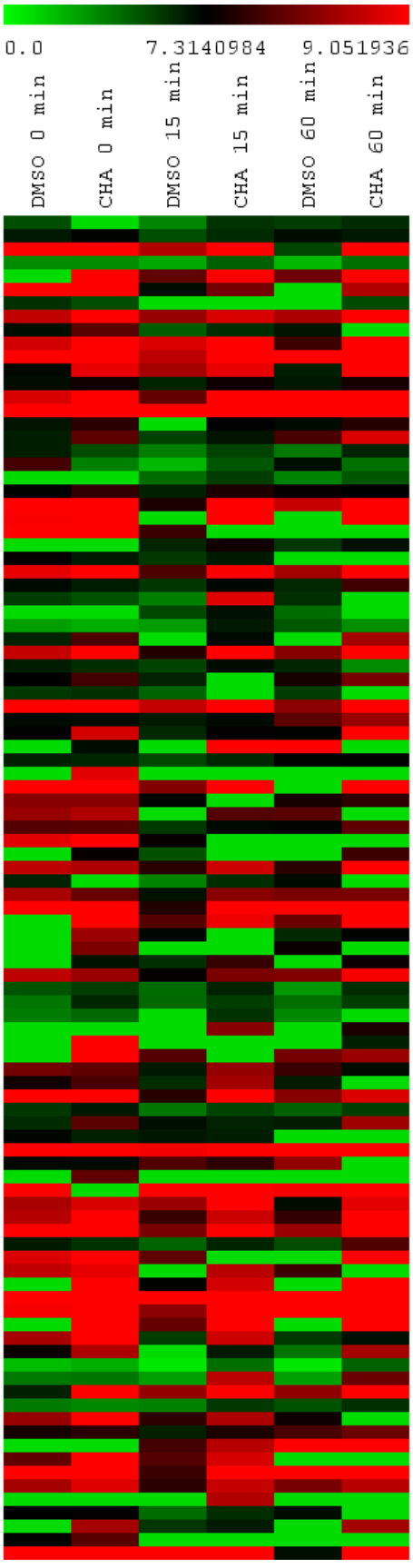
KIF26P

G)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C11orf91
C11orf91
ZNF783
CCDC180
SLFN12L
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L0C102723805
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L0C102724257
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CD68
CFAP43
CRAT
CSF2RB
gag-pro-pol
GLTP
G0LGA6L1
IFT122
IFT172
L0C100873985
L0C440157
MLH1
MMRN2
MT-ND4
MT-ND5
MYLK2
NAP1L3
NBPF9
NEBL
NME4
P0LR3G
PSPHP1
RAF2
REM2
SUPT5H
TAS2R45
WDR63
Crystal Structure 0f Alpha-1-Antitrypsin
Structural Insights Into Nedd8 Activation 0f Cullin-Ring
unnamed protein product
unnamed protein product
pro protein
immunoglobulin lambda light chain
subl.5
unnamed protein product
"inhibitor BrCN fragment II,alphal prote"
"B cell antibody heavy chain variable region, partial "
unnamed protein product
"immunoglobulin light chain variable region, partial "
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immunoglobulin heavy chain variable region
Chromosome 18 open reading frame 56
unnamed protein product
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immunoglobulin M heavy chain variable region
unnamed protein product
unnamed protein product
unnamed protein product
"GDF5, partial "
ATPase
MHC class II antigen
unnamed protein product
immunoglobulin gamma 1 heavy chain variable region
Crystal Structure 0f Phosphorylated P38 Alpha
Crystal Structure 0f The Homeobox Domain
immunoglobulin heavy chain variable region (VDJ)
unnamed protein product
immunoglobulin heavy chain
"Chain A, Crystal Structure 0f Topbpl Brct78"
tospeak-10
immunoglobulin variable region
HFSE-1
anti-HIV-1 immunoglobulin heavy chain variable region
anti-HIV-1 immunoglobulin heavy chain variable region
unnamed protein product
unnamed protein product
unnamed protein product
unnamed protein product
unnamed protein product
unnamed protein product
unnamed protein product
unnamed protein product
unnamed protein product
anti-tetanus toxoid immunoglobulin light chain
anti-tetanus toxoid immunoglobulin light chain
Crystal Structure 0f Arc4 From Human Tankyrase 2
"immunoglobulin G heavy chain variable region, partial "
"MHC class I antigen, partial "
immunoglobulin heavy chain variable region
Crystal Structure 0f Human Pacsin 1 F-Bar Domain
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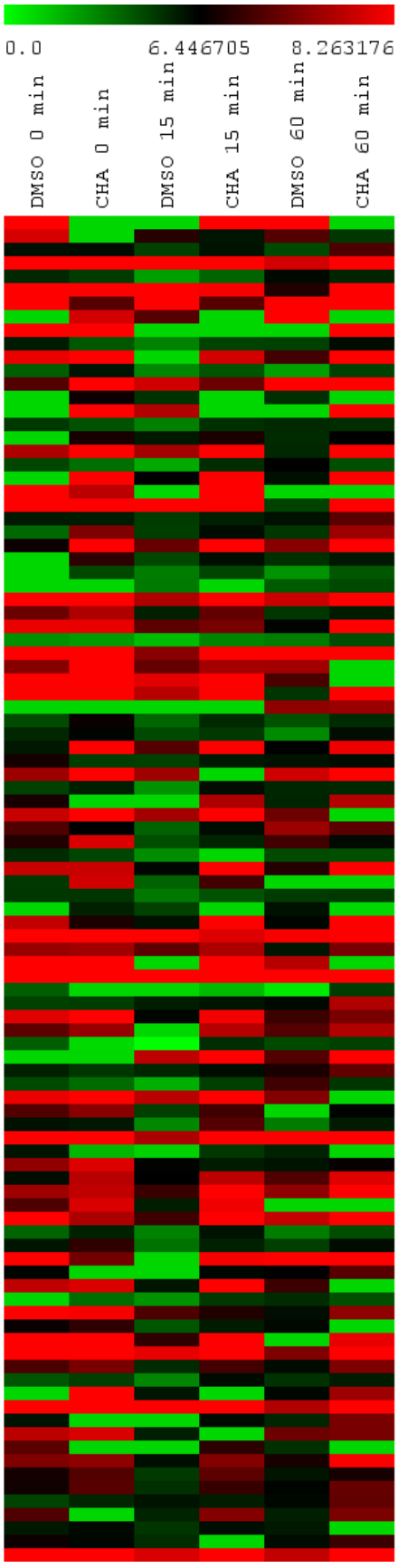
NADH dehydrogenase subunit 4L (mitochondrion)
"Chain B, Hla-Dr1 With Covalently Linked Clip106-120"
immunoglobulin heavy chain variable region
unnamed protein product

H)



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unnamed protein product
T-cell receptor beta chain
Revealing A Marine Natural Product
unnamed protein product
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Structure Of A Complex Between Ccps 6 And 7
X-ray Structure Of Alkaline Phosphatase
"Chain D, Human SdhNADHINHIBITOR COMPLEX"
"Chain C, Structural Mechanisms Of Camptothecin"
putative uncharacterized protein FLJ44967-like
cytochrome c oxidase subunit I (mitochondrion)
ATP synthase F0 subunit 6 (mitochondrion)
"monoclonal antibody CH94 heavy chain, partial "
"Chain H, 14-3-3gamma Complexed"
immunoglobulin kappa chain variable region
MHC class II antigen
unknown protein
unnamed protein product
unnamed protein product
"Chain H, Crystal Structure Of High-affinity Von Willebrand'
LOW QUALITY PROTEIN: spidroin-1-like
RNA-binding protein 6-like isoform X8
speedy protein E2-like
putative COBW domain-containing protein 7-like
unnamed protein product
immunoglobulin epsilon heavy chain variable region
immunoglobulin kappa light chain variable region
immunoglobulin alpha heavy chain variable region
LDLR-FUT fusion protein
"Chain A, Gppnhp-Bound Rab6 Gtpase"
immunoglobulin lambda light chain variable region
ADP-ribosylation factor
unnamed protein product
"Chain A, Crystal Structure Of Human Placental Lactogen"
"Chain A, Human Alpha-Phosphomannomutase 1"
CDS feature is included to show the translation
unnamed protein product
"Chain C, Structure Of Human Ubiquitin-Conjugating Enzyme"
hypothetical protein
unnamed protein product
immunoglobulin heavy chain variable region
immunoglobulin heavy chain variable region
immunoglobulin heavy chain variable region
immunoglobulin heavy chain variable region
"Chain A, Structure And Mechanism Of Interleukin-1beta"
unknown protein
Crystal Structure Of Human Glutathione-S-Transferase
hCG2029550
"chromosome 6 open reading frame 54, isoform CRA_a "
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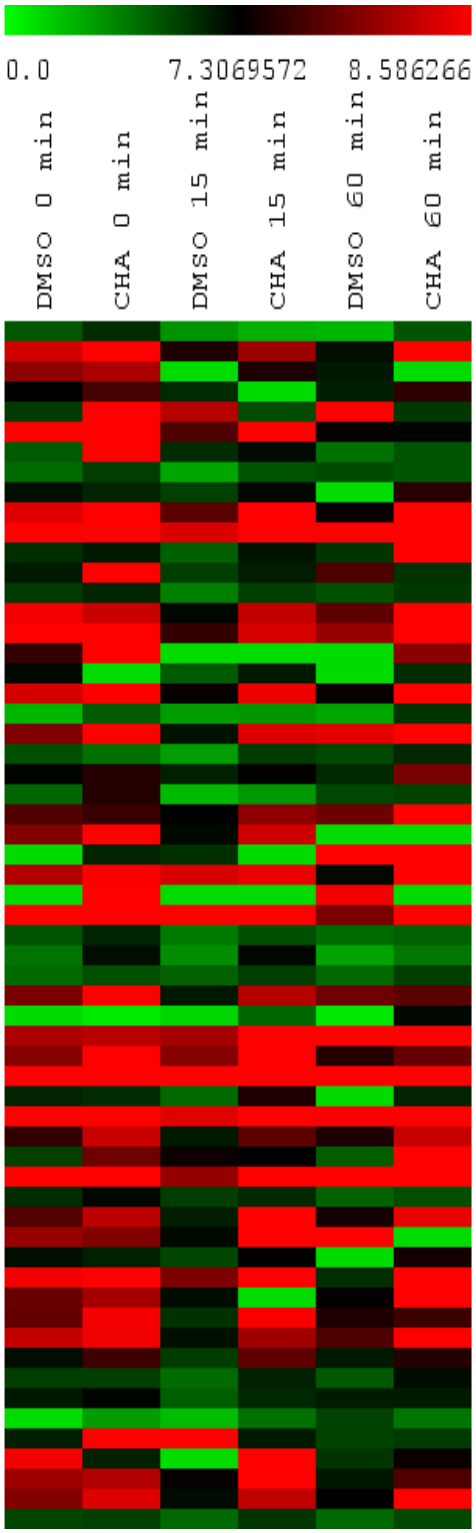
I)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"hCG1641491, isoform CRA_d "
"hCG1786642, isoform CRA_b "
"hCG1644277, isoform CRA_b "
chromosome 14 open reading frame 162
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hCG1658585
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hCG2040546
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"hCG1814474, isoform CRA_a "
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hCG2038840
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hCG1643292
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"hCG1787701, isoform CRA_b "
"hCG1750329, isoform CRA_a "
"hCG2019008, isoform CRA_d "
hCG2045838
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MHC class I antigen
unnamed protein product
Crystal Structure Of The Human Natural Killer Cell
unnamed protein product
unnamed protein product
Crystal Structure Of The Human Rhoc-gppnhp
Crystal Structure Of The First Fibronectin Type Iii
X-Ray Crystal Structure Of Putative Human Ras
Structure Of Human Pdlim5
T cell receptor
immunoglobulin heavy chain variable region
7B2 protein
T cell receptor beta variable 5
"Chain A, Structure Of E163kR145E DJ-1"
The Crystal Structure Of P18
"anti-DNA immunoglobulin heavy chain, partial "
unknown

VITA

My name is Miss Orapan Parnsukhon was born in Chachoengsao, Thailand on September 4, 1989. After graduation with the Bachelor's degree of Science from Department of Microbiology, Faculty of Science at Burapha University in 2009. I subsequently enrolled in the Master's degree of Program in Industrial Microbiology, Faculty of science at Chulalongkorn University in 2010.

Academic presentation

Parnsukhon, O., Palaga, T., Yingyongnarongkul, B. and Yompakdee, C. Anti-proliferation activity of a new coumarin compound from Clausena harmandiana on human cancer cell lines. The 25th Annual Meeting of the Thai Society for Biotechnology and International Conference: "Agro-Industrial Biotechnology for Global Sustainable Prosperity" 2013 October 16-19, The Emerald Hotel, Bangkok, Thailand.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University