Complete report

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Molecular target of an anti-cancer compound from leaves of *Clausena harmandiana* (Pierre)

by

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บทคัดย่อ

Clausena harmandiana (Pierre) Guillaumin หรือ ส่องฟ้าดง จัดอยู่ในวงศ์ Rutaceae ในงานวิจัยก่อนหน้า ได้แยกสารบริสุทธิ์จากส่วนใบของ *C. harmandiana* พบว่าเป็นสารประกอบคูมาริน ชนิดหนึ่งโดยให้ชื่อว่า CHA-01 มีฤทธิ์ยับยั้งวิถีการส่งสัญญาณของแคลเซียมในยีสต์ *Saccharomyces cerevisiae* สายพันธุ์กลายที่ขาดยีน *ZDS1* (delta *zds1*) แต่ความรู้เกี่ยวกับฤทธิ์ทางชีวภาพของสารประกอบคูมาริน CHA-01 ยังมีอยู่น้อยมาก เนื่องจากเคยมีรายงานว่าสารประกอบคูมารินอื่นๆบางชนิด มีฤทธิ์ต้านมะเร็ง จึงนำมาสู่วัตถุประสงค์ของงานวิจัยนี้คือเพื่อที่จะศึกษากลไกในระดับโมเลกุลของสาร CHA-01 ในการต้านการเพิ่มจำนวนของเซลล์ไลน์มะเร็งชนิด Jurkat ผลการทดลองพบว่า CHA-01 มีฤทธิ์ต้านการเพิ่มจำนวนของเซลล์ไลน์มะเร็งหลายชนิด ได้แก่ เซลล์ไลน์ Jurkat (เซลล์ไลน์มะเร็งเม็ดเลือดขาวชนิดลิมโฟไซท์), KATO III (เซลล์ไลน์มะเร็งกระเพาะอาหาร) และ THP1(เซลล์ไลน์มะเร็งเม็ดเลือดขาวชนิดมอนอไซท์) โดยวิธี MTT assay โดยพบว่าเซลล์ไลน์มะเร็งชนิด Jurkat มีความไวต่อ CHA-01 มากที่สุด มีค่า IC_{50} เท่ากับ 0.67ไมโครโมลาร์ และไม่มีความเป็นพิษต่อเซลล์เม็ดเลือดขาวชนิดลิมโฟไซท์ของคนปกติที่ความเข้มข้นสูงถึง 10 ไมโครโมลาร์ จากการวิเคราะห์โดยวิธี Flow cytometry พบว่า CHA-01 ทำให้วัฏจักรเซลล์หยุดอยู่ที่ระยะ S อันเป็นผลเนื่องมาจากไปยับยั้งการสังเคราะห์ DNA นอกจากนี้ CHA-01 ยังสามารถชักนำให้เซลล์มะเร็งตายแบบอะพ็อพโทซิส ผลการทดลองเหล่านี้ชี้ให้เห็นบทบาทของ CHA-01 ที่เกี่ยวกับฤทธิ์ต้านมะเร็งโดยเฉพาะมะเร็งเม็ดเลือดขาวชนิดลิมโฟไซท์ การทดลองต่อไปจะใช้การวิเคราะห์ทางโปรติโอมิกส์เพื่อศึกษาผลของ CHA-01 ที่มีต่อระดับการแสดงออกของโปรตีนทั้งหมดในเซลล์ไลน์มะเร็งชนิด Jurkat

Abstract

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* (delta *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 aim of this research was to study molecular mechanism on anti-proliferation activity of CHA-01 in Jurkat T cells. The results revealed that CHA-01 showed anti-proliferative activity in several cell lines including Jurkat (Lymphocytic leukemic cell line), KATO III (Stomach cancer cell line) and THP1 (Monocytic leukemic 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 μM. It contained no cytotoxic activity against normal lymphocytes up to 10 μM. Flow cytometric analysis revealed that the CHA-01 caused cell cycle arrest at S phase in Jurkat T cells as a result from inhibition of DNA synthesis. Moreover, CHA-01 induced apoptotic cell death in the Jurkat T cells.

Our results revealed the role(s) of CHA-01 on its anti-cancer activity especially against lymphocytic leukemia. Future study will utilize proteomic analysis on expression level of proteins in CHA-01 treated Jurkat T cells.

Introduction

Cancer is still one of the leading causes of morbidity and mortality worldwide. Globally, the number of cancer deaths is projected to increase from 7.1 million in 2002 to 11.5 million in 2030 (Mathers and Loncar, 2006). Chemotherapy is routinely used for treatment of cancer. Since cancer cells lose many of the regulatory functions present in normal cells, they continue to divide when normal cells do not. This feature makes cancer cells susceptible to chemotherapeutic drugs. However, chemotherapeutic treatments are not devoid of their own intrinsic problems. Various kinds of toxicities may occur as a result of chemotherapeutic treatments (Desai et al. 2008).

The anticancer properties of plants have been recognized for centuries. The anticancer characteristics of a number of plants are still being actively researched and some have shown promising results were reviewed in Desai et al. 2008.

Clausena 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*. Suauam et al. (2015) examined the bioactive and pure compounds from *C. harmandiana* by Δ*zds1* proliferation assay and found that a coumarin compound designated CHA-01 exhibited calcium signaling pathway inhibition in Δ*zds1* yeast strain. Furthermore experiments revealed that the coumarin compound, CHA-01, can inhibit calcineurin phosphatase activity and is a potential immunosuppressant (Suaum et al. 2015). Coumarins naturally present in many plants and have been reported to exhibit various biological activities including anticancer, anti-inflammatory, anticoagulant, antimicrobial, antioxidant and anti-allergic properties (Wu et al. 2009, Riveiro et al. 2010). Certain coumarin compounds could inhibit the cell growth in various types of cancer cell lines (Marshall et al. 1994, Chuang et al. 2007).

Proteomics is the large-scale study of proteins, particularly in clinical and pharmacological study. The potential biological roles and functions of individual proteins could be determined by proteomics approaches (Booranasrisak et al. 2013). Especially in

cancer research, pathway and proteome study with anticancer drugs and drug candidates is widely investigated using proteomic techniques on the protein level.

In this research aimed to investigate the role of the coumarin compound (CHA-01) on anti-cancer activity and then proteomics approach was used to improve the understanding of the underlying anti-cancer molecular mechanisms of the coumarin CHA-01 on lymphocytic leukemia.

Materials and methods

Source of CHA-01

A pure coumarin, CHA-01, isolated and purified from leave extract of *C. harmandiana* (Pierre), was obtained from Assoc. Prof. Dr. Boonek Yingyongnarongkul, Department of Chemistry, Faculty of Science, Ramkamhaeng University. The stock solution of CHA-01 was prepared in dimethyl sulfoxide (DMSO) (Amresco, USA).

Cell line and cultivation

Jurkat T cell (Human acute T cell leukemia), THP-1 (Human acute monocytic leukemia) and KATO III (Human gastric carcinoma) were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Thailand. Cells were grown in RPMI-1640 with L-Glutamine (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone, USA), 10^6 U/ml penicillin (General Drugs House Co. Ltd., Bangkok, Thailand) and 500 mg/ml streptomycin (AppliChem, USA). The cells were cultured at 37°C in 5% CO_2 containing atmosphere. Normal human blood cells were obtained from the Thai Red Cross Society.

Preparation of human lymphocytes

Human normal lymphocytes were isolated from whole blood by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare Bio-Sciences, UK) by following the manufacturer's instruction.

The cytotoxic effect of the CHA-01 on human normal lymphocytes was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, the test 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 (0.001, 0.01, 0.1, 1, 10, 100 μM). For a control experiment, the cells were treated with DMSO. After 1 or 4 days of

incubation at 37°C, 5% CO_2, 10 µl of 5 mg/ml MTT solution (Bio Basic Inc, Canada) was added and incubated further for 4 h. Then, 100 µl of isopropanol was added to dissolve the colored formazan crystal produced from MTT. Viability of cells was measured at 540 nm by a microplate reader (ELx800 Bio-tek instrument, USA)

MTT Proliferation assay

The cytotoxic effect of the CHA-01 on human cancer cell lines was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). 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). For non-adherent lines (Jurkat T cell and THP-1 cell), cells were directly incubated with the compounds. For a control experiment, the cells were treated with DMSO and incubated at 37°C in the atmosphere of 5% CO_2 for 1 or 4 days. After 1 or 4 days of incubation at 37°C in the atmosphere of 5% CO_2, 10 µl of 5 mg/ml MTT solution (Bio Basic Inc, Canada) was added and incubated further for 4 h. Then 100 µl of 0.04 N HCl (Merck, Germany) in isopropanol (Merck, Germany) was added to dissolve the colored formazan crystal produced from MTT. Viability of cells was measured at 540 nm by a microplate reader (ELx800 Bio-tek instrument, USA).

Flow cytometric analysis

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 concentration of 1 µM and the plates were incubated at 37°C, 5% CO_2 for 24 h. Cells were centrifuged at 4,200 × *g* for 5 min. The pellets were rinsed twice with phosphate-buffered saline (PBS) and fixed using cold 70% ethanol for overnight at 37°C. Then, the cells were incubated with 10 mg/ml RNase A (Sigma, USA) at 37°C for 30 min. The cells were then stained with 1 mg/ml propidium iodide (Sigma, USA) for 30 min at 37°C in the dark. The samples were analyzed on a flow cytometer (Beckman Coulter, USA).

Apoptosis analysis

One µM of CHA-01 A was added into wells with a total of 2×10^6 cells/well of Jurkat T cells. Cells were incubated at 37°C, 5% CO_2 for 6, 12 and 24 h, respectively. After harvesting and washing, 200 µL cell suspension was added with 3 µl of the annexin

conjugate ApopNexin™ FITC and 2 µl of 100X Propidium Iodide (PI). 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.

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.

Results and discussion

CHA-01 exhibited anti-proliferation activity against several human cancer cell lines

The cytotoxic effect of CHA-01 on Jurkat T cell, KATTO III and THP-1 cell lines were 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. As shown in Fig.1a, b and c, the CHA-01 showed cytotoxic effects against Jurkat T cell, KATO III and THP-1 when compared with control. The inhibition of cell proliferation was in a dose- and time-dependent manner. CHA-01 did not showed acute cytotoxicity effects against Jurkat T cell, KATO III and THP-1 for 1 day but caused chronic cytotoxicity effects after incubated for 4 days. The IC_{50} values of the CHA-01 in Jurkat T cell, KATO III and THP-1 after incubation for 4 days were 0.67, 1.06 and 1.05 μM, respectively. These results indicated that Jurkat T cell was the most sensitive to the cytotoxic effect of CHA-01 among the three cell lines. Therefore, we chose Jurkat T cell, the most CHA-01 sensitive cell line, for further study.

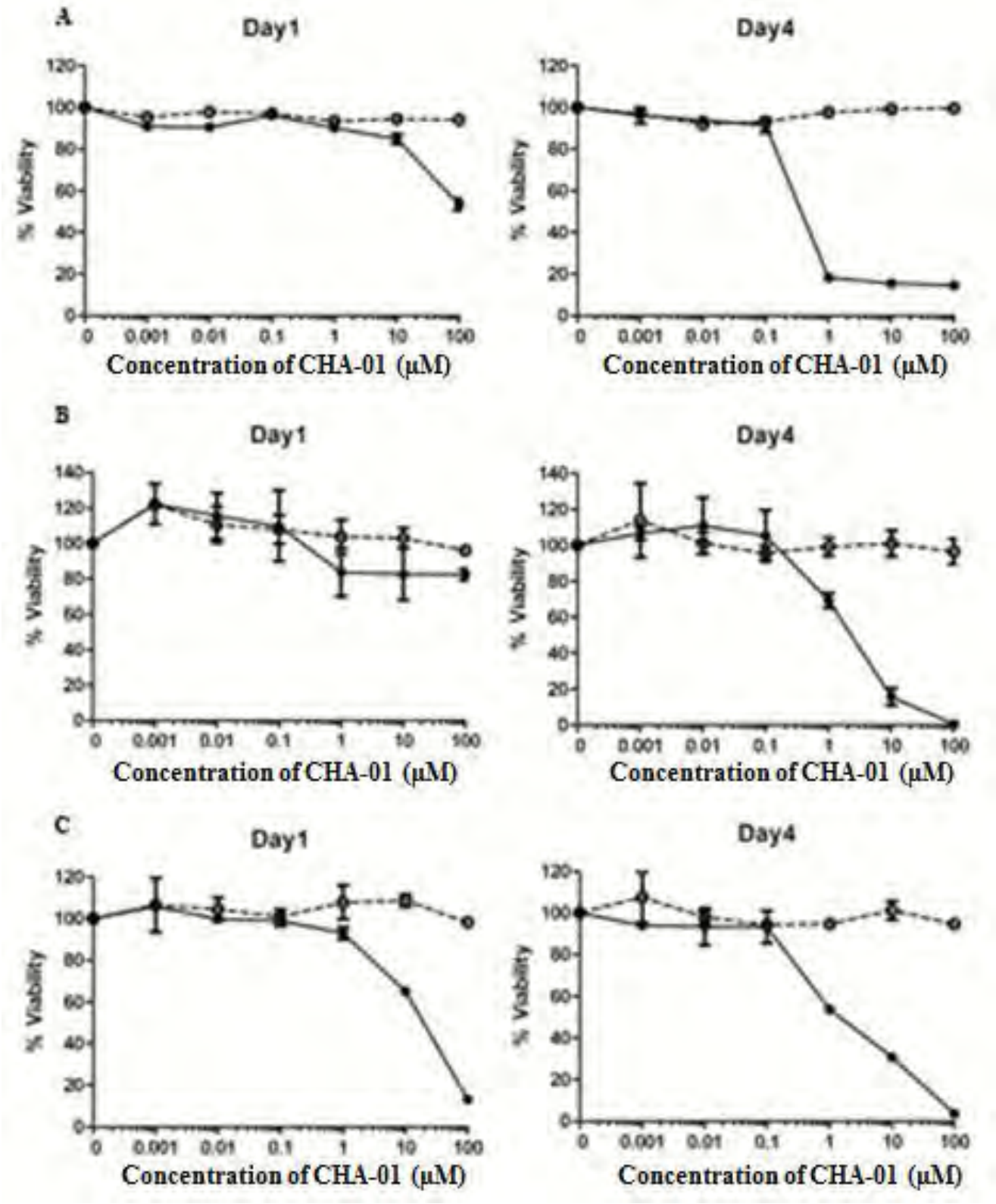


Figure 1 Cytotoxicity of CHA-01 on human cancer cell lines was assessed using MTT assays. Cells (Jurkat T cell (a) , KATTO III(b) and THP-1(c)) were treated with 0.001, 0.01, 0.1, 1, 10, 100 μM of the CHA-01 compared with control (○) incubated at 37°C in the atmosphere of 5% CO_2 for 1 day or 4 days, respectively. The data at each time point were mean ± SD from triplicate repeats.

No cytotoxic effect of CHA-01 on normal human lymphocytes

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 on normal lymphocytes or not. To test the hypothesis, normal lymphocytes isolates from normal blood donors were treated with different doses (from 0.001-100 μM) of CHA-01 for 1 and 4 days. Interestingly, CHA-01 showed much less toxicity to normal lymphocytes than the Jurkat T cells. For day 4, percentage of viability decreased to lower than that of day 1 due to life span of the lymphocytes. Only at a dose of 100 μM that CHA-01 showed acute cytotoxic effect to normal white blood cells (Fig. 2).

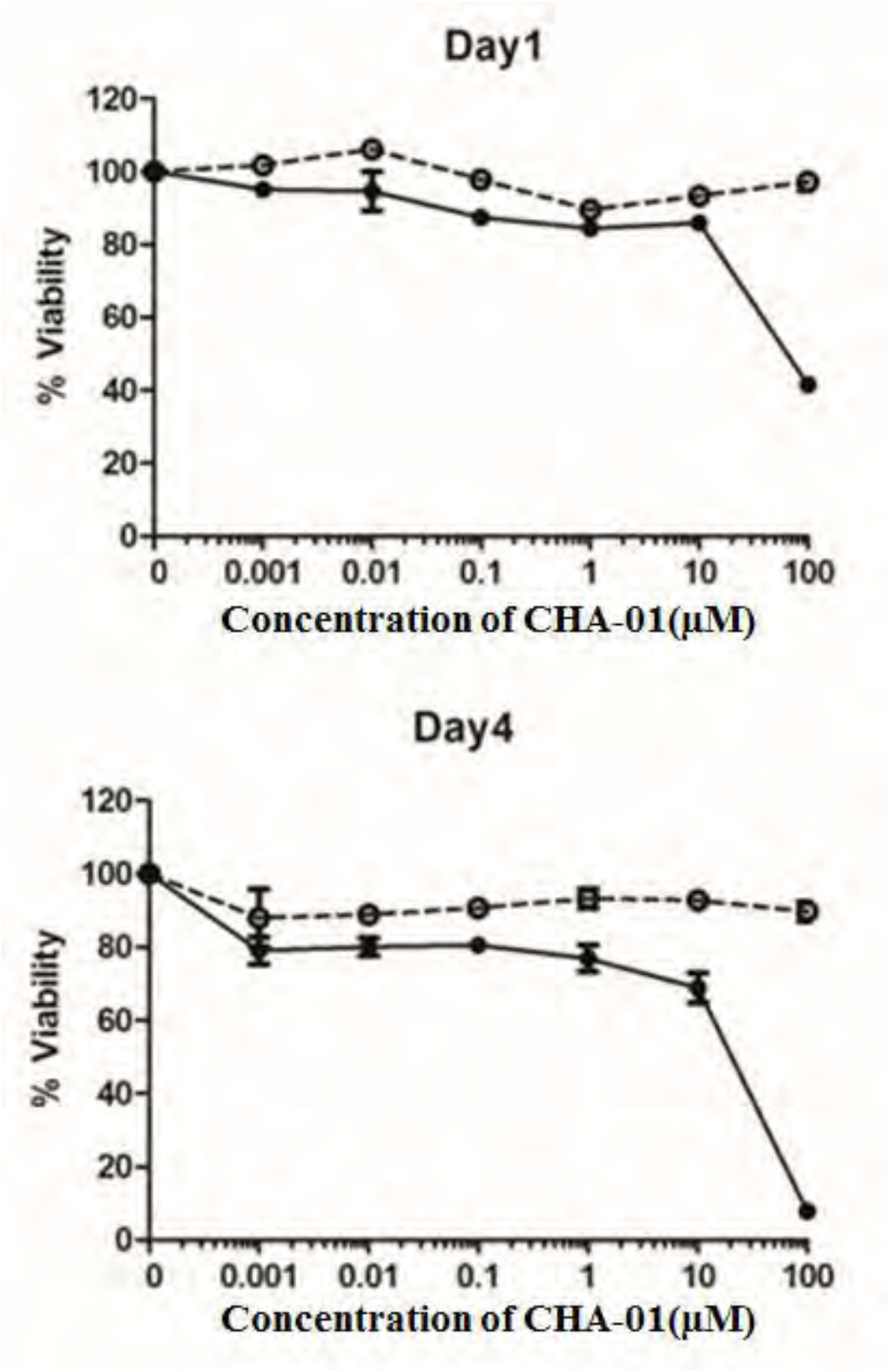


Figure 2 Cytotoxicity of CHA-01 on normal human white blood cells was assessed using MTT assays. Cells were treated with 0.001, 0.01, 0.1, 1, 10, 100 μM of the CHA-01 (●) compared with control (○)incubated at 37°C in the atmosphere of 5% CO_2 for 1 day or 4 days, respectively. The data at each time point were mean ± SD from triplicate repeats.

CHA-01 induced S phase cell cycle arrest in Jurkat T cells

To address whether CHA-01 effected cell cycle and to determine the mechanism for the antiproliferative effect of CHA-01, DNA content of Jurkat T cells treated for 24 h with or without 1 μM CHA-01 were analyzed by flow cytometry. Results showed that CHA-01 treatment caused an increase in the population of S phase cells from 17.45 % in the control treatment to 24.75% in the cells treated with 1 μM CHA-01. Furthermore, an increase in the population of Sub G_1 phase cells from 2.5% in the control treatment to 9.4% in the cells treated with 1 μM CHA-01 were observed. Results suggested that Jurkat T cells treated with CHA-01 arrested in S phase of the cell cycle and may lead to apoptosis (Fig 3).

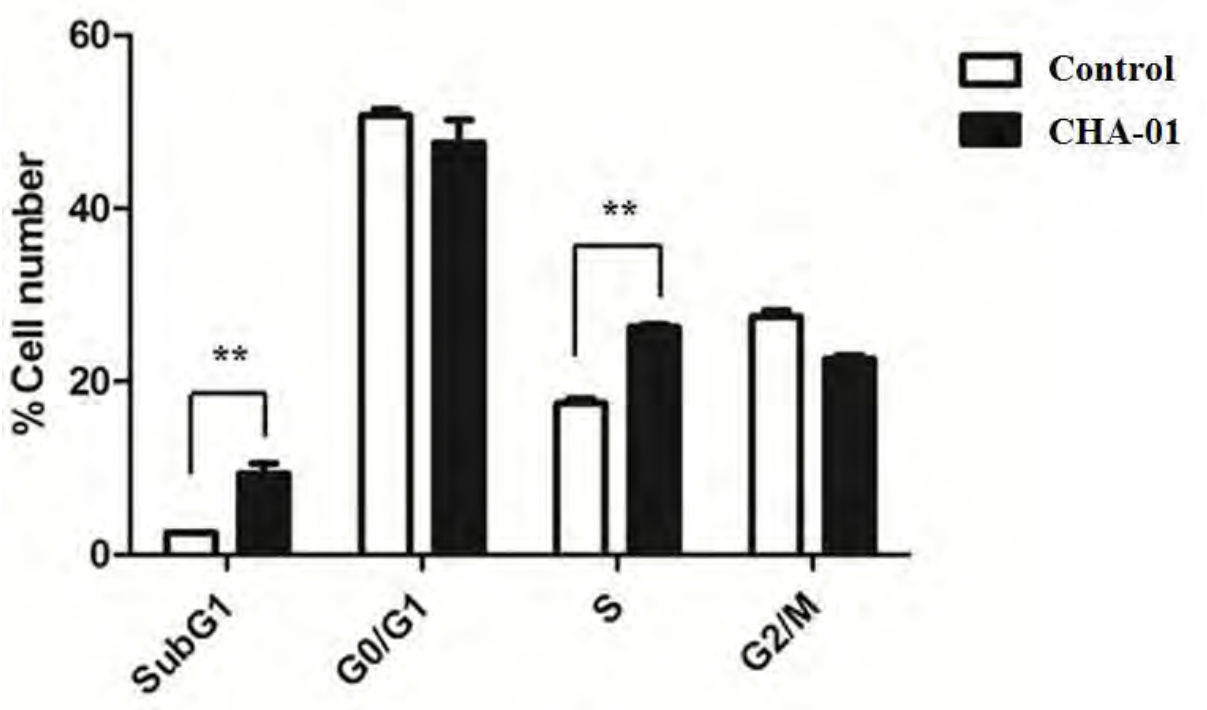


Figure 3 Effect of CHA-01 on cell cycle distribution in Jurkat T cells. The cells were incubated for 24 h at 37°C in the atmosphere of 5% CO_2 in the absence (control) or presence of 1 μM CHA-01, after which the DNA content was determined by flow cytometry. The data showed the values of mean ± SD from the triplicate experiments. ** Significant differences at *p*-value <0.01

CHA-01 induces apoptosis in Jurkat T cell

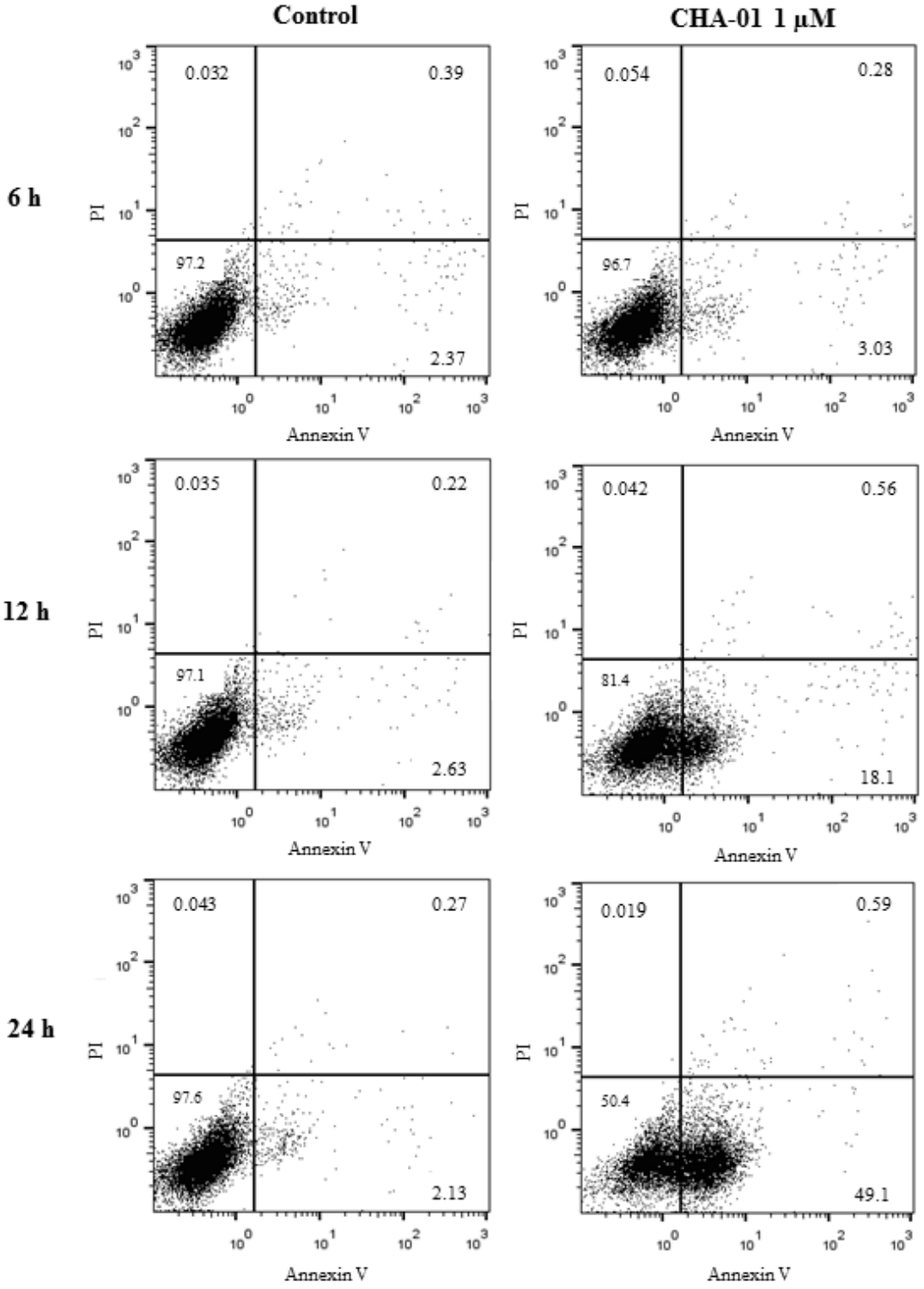
Due to the clearly increase in sub G_1 cells caused by 1 μM CHA-01 treatment in Jurkat T cells, we wondered whether CHA-01 could induce apoptosis in Jurkat T cells. Jurkat T cells were incubated with 1 μM CHA-01 for 6, 12 and 24 h, respectively. After Annexin V-FITC and PI staining, flow cytometry analysis 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. 4A) 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. 4E). While in controls, early apoptosis rate were 2.63% and 2.13% for 12 and 24 h, respectively (Fig. 4A, B). The percentage of viable cells was significantly decreased (81.4% and 50.4% for 12 and 24 h) (Fig. 4D), 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. 4B, 4C). The results clearly indicated that CHA-01 could induce apoptosis.

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

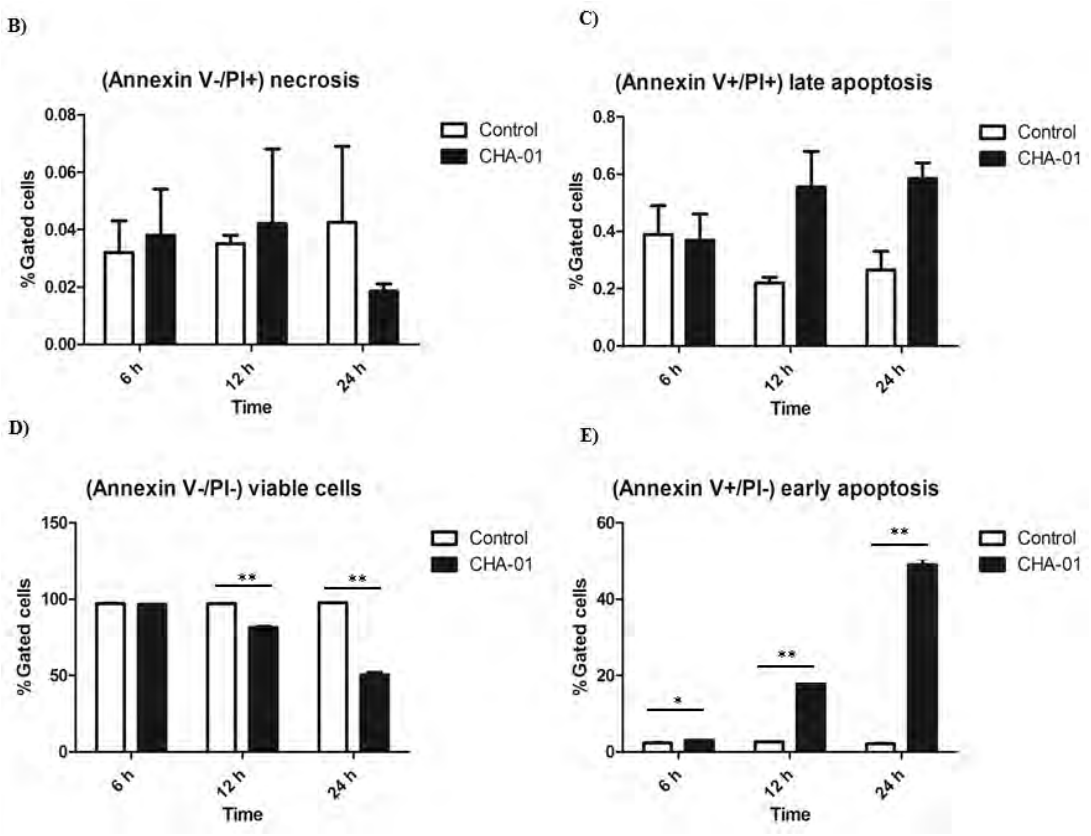


Figure 4. CHA-01 induced apoptosis in Jurkat T cell. The cells were incubated for 6, 12, 24 h in the absence (control, DMSO) or presence of 1 μM CHA-01 at 37°C in the atmosphere of 5% CO_2 (A) Original dot plots of the PI versus the Annexin V-FITC fluorescence intensities. Annexin V-FITC negative/PI 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.

CHA-01 inhibits DNA synthesis

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. 5)

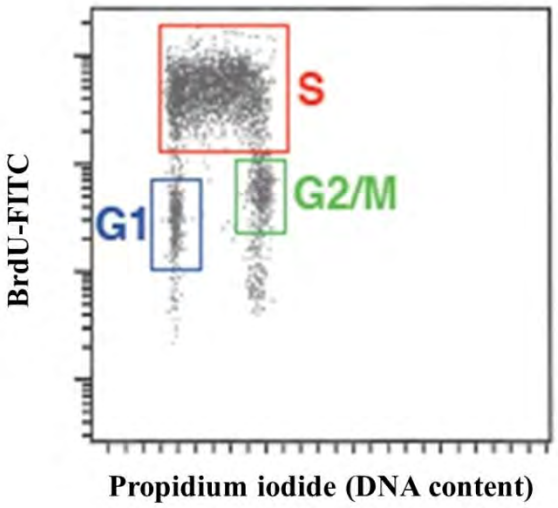


Figure 5. Typical bivariate plot showing DNA content (propidium iodide, X axis) and BrdU incorporation (FITC staining, Y axis). The G1, S and G2/M phase of the cell cycle can be better delineated (Ryba et al. 2011).

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. 6. 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. 7). These results suggested that CHA-01 possesses a DNA synthesis inhibiting activity.

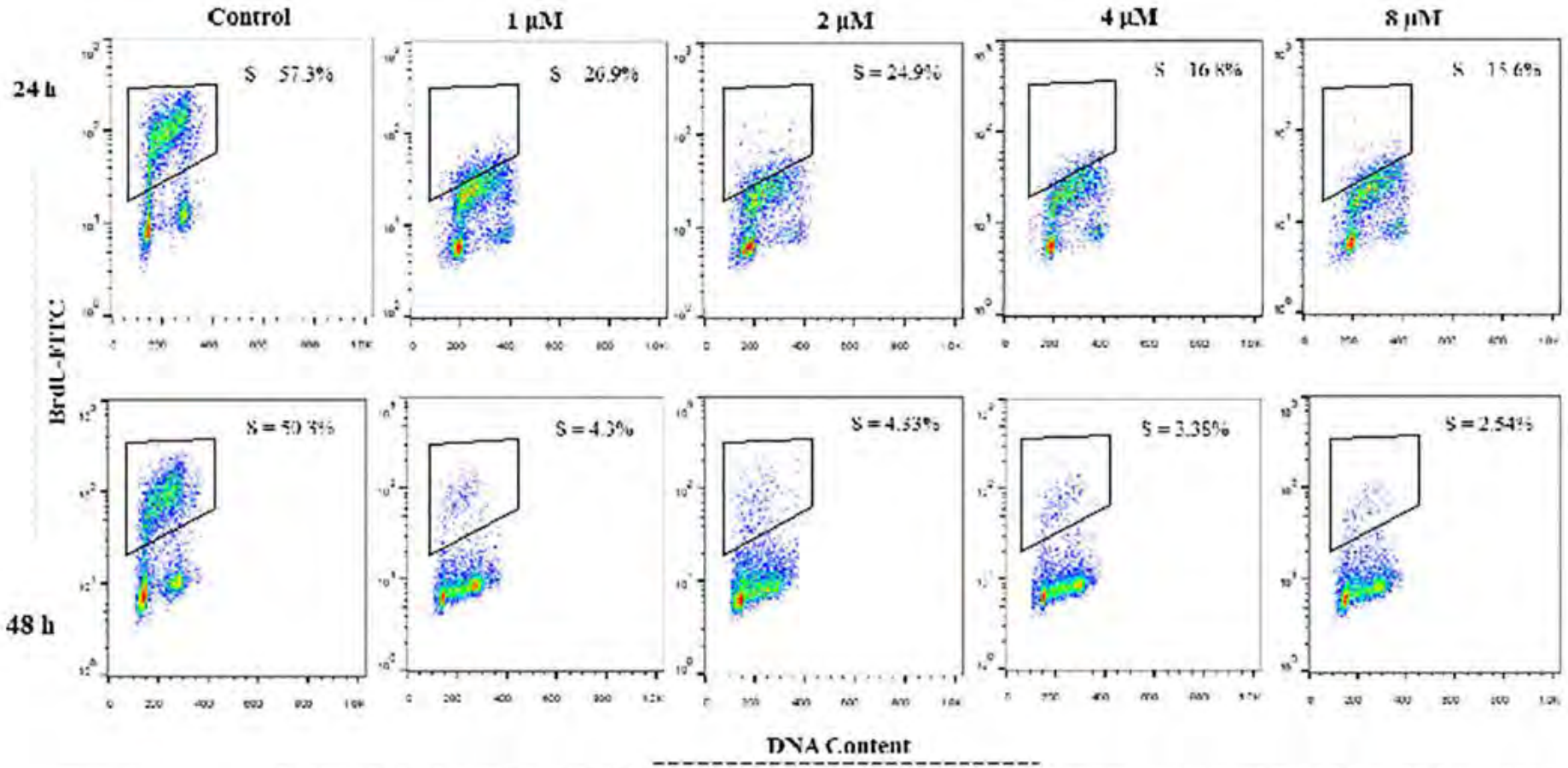


Figure 6 Effect of DNA synthesis inhibitors on BrdU incorporation. Jurkat T cells were treated for 24 h or 48 h at 37°C in the atmosphere of 5% CO_2 without or with 1, 2, 4 or 8 μM Clausmarin A and the cells were incubated for 1 h with BrdU and analyzed by flow cytometer. S: S phase cells.

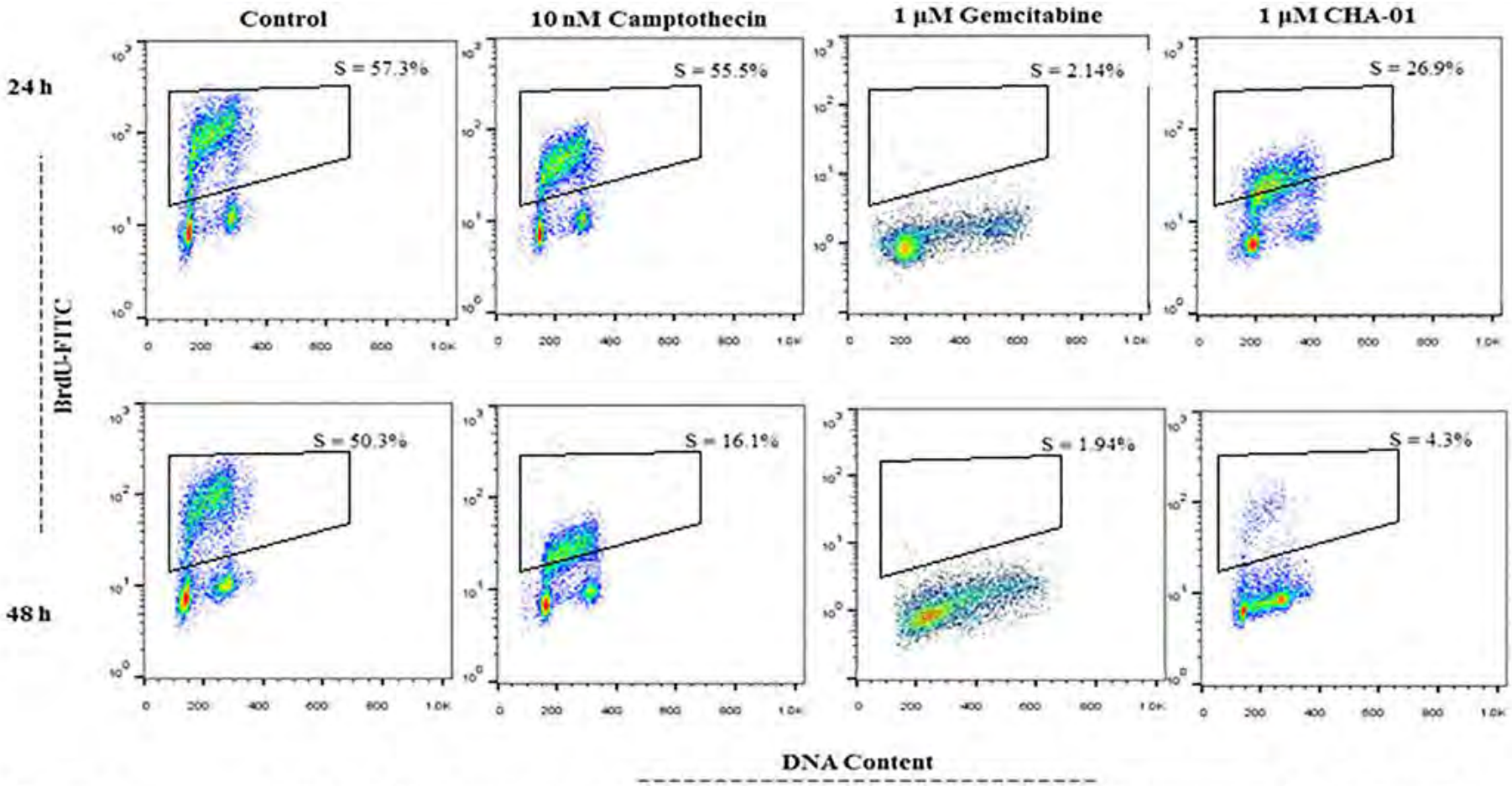


Figure 7 Effect of DNA synthesis inhibitors on BrdU incorporation. Jurkat T cells were treated for 24 h or 48 h at 37°C in the atmosphere of 5% CO_2 with 10 nM camptothecin, 1 μM gemcitabine and1 μM CHA-01 and incubated for 1 h with BrdU and analyzed by flow cytometry. S: S phase cells

Conclusion

Our study showed that a new coumarin compound, CHA-01, from the leave extract of C. *harmandiana* (Pierre) exhibited cytotoxic effect against Jurkat T cells without causing cytotoxicity to normal lymphocytes. We further found that CHA-01 induced cell cycle arrest at S phase in the Jurkat T cell line as a result of inhibition of DNA synthesis. CHA-01 itself could induce apoptosis

Future perspective

To understand more on molecular mechanism of CHA-01 on Jurkat T cells that involved in anti-proliferation activity, proteomic analysis of CHA-01 A treated Jurkat T cells will be investigated. Detail data analysis using several software programs or several analysis tools will be carried out to obtain the candidate proteins. Genes encoding the candidate proteins will be cloned. Expression level of the candidate genes in response to CHA-01 treatment will be carried out by Real-time RT-PCR. A model will be proposed for molecular mechanism of CHA-01 on anti-proliferation activity in Jurkat T cells.

Acknowledgement

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References

Booranasrisak, T., N. Phaonakrop, J. Jaresitthikunchai, C. Virunanon, S. Roytrakul and W. Chulalaksananukul. 2013. Proteomic evaluation of free fatty acid biosynthesis in Jatropha curcas L. (physic nut) kernel development. **Afr. J. Biotechnol.** 12(21): 3132-3142.

Casciola-Rosen, L., A. Rosen, M. Petri and M. Schlissel (1996). Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: Implications for coagulation events and antigenic spread in systemic lupus erythematosus. **Proc. Natl. Acad. Sci. USA** 93: 1624-1629.

Chuang, J. Y., Y. F. Huang, H. F. Lu, H. C. Ho, J. S. Yang, T. M. Li, N. W. Chang and J. G. Chung. 2007. Coumarin induces cell cycle arrest and apoptosis in human cervical cancer HeLa cells through a mitochondria- and caspase-3 dependent mechanism and NF-*κ*B down-regulation. **In vivo.** 21: 1003-1010.

Darzynkiewicz, Z., G. Juan, X. Li, W. Gorczyca, T. Murakami and F. Traganos (1997). Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (nCytometryecrosis). **Cytometry** 27: 1-20.

Dean, P. N., F. Dolbeare, H. Gratzner, G. C. Rice and J. W. Gray (1984). Cell-cycle analysis using a monoclonal antibody to BrdUrd. Cell Tissue Kinet 17(4): 427-436.

Desai, A.G., G.N. Qazi, R.K. Ganju, M. El-Tamer, J. Singh, A.K. Saxena, Y.S. Bedi, S.C. Taneja, and H.K. Bhat. 2008. Medicinal Plants and Cancer Chemoprevention. **Curr. Drug Metab.** 9(7): 581–591.

Gratzner, H. G. and R. C. Leif (1981). An immunofluorescence method for monitoring DNA synthesis by flow cytometry. **Cytometry** 1(6): 385-389.

Lacombe, F., F. Belloc, P. Bernard and M. R. Boisseau (1988). Evaluation of four

methods of DNA distribution data analysis based on bromodeoxyuridine/DNA bivariate data. **Cytometry** 9: 245-253.

Majno, G. and I. Joris (1995). Apoptosis, oncosis, and necrosis an overview of cell death. **Am. J. Pathol.** 146(1): 3-15.

Maneerat, W., W. Phakhodee, T. Ritthiwigrom, S. Cheenpracha, T. Promgool, K. Yossathera, S. Deachathai and S. Laphookhieo. 2012. Antibacterial carbazole alkaloids from Clausena harmandiana twigs. **Fitoterapia.** 83: 1110-1114.

Marshall, M. E., K. Kervin, C. Benefield, A. Umerani, S. Albainy-Jenei, Q. Zhao and M. B. Khazaeli. 1994. Growth-inhibitory effects of coumarin (1,2-benzopyrone) and 7-hydroxycoumarin on human malignant cell lines in vitro. **J. Cancer Res. Clin. Oncol.** 20 (Suppl): S 3 - S 10.

Mathers, C.D., D. Loncar. 2006. Projections of Global Mortality and Burden of Disease from 2002 to 2030. **PLoS Med.** 3(11): e442. doi: 10.1371/journal.pmed.

Miltenburger, H. G., G. Sachse and M. Schliermann (1987). S-phase cell detection with a monoclonal antibody. **Dev. Biol. Stand.** 66: 91-99.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **J. Immunol. Methods.** 65:55-63.

Suauam, P., B. Yingyongnarongkul, T. Palaga, T. Miyakawa and C. Yompakdee. 2015. Clausmarin A, potential immunosuppressant revealed by yeast-based assay and interleukin-2 production assay in Jurkat T cells. **PLoS ONE.** 10(8). 1-14.

Riveiro, M.E., N. De Kimpe, A. Moglioni, R. Vázquez, F. Monczor, C. Shayo and C. Davio. 2010. Coumarins: Old compounds with novel promising therapeutic perspectives. **Curr. Med. Chem.** 17: 1325–1338.

Ryba, T., D. Battaglia, B. D. Pope, I. Hiratani and D. M. Gilbert (2011). Genome-scale analysis of replication timing: from bench to bioinformatics. **Nat. Protoc.** 6(6): 870-895.

Wu, L., X. Wang, W. Xu, F. Farzaneh and R. Xu. 2009. The structure and pharmacological functions of coumarins and their derivatives. **Curr. Med. Chem.** 16: 4236–4260.