Inhibition of Hedgehog signaling induces monocytic differentiation of HL-60 cells

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Inhibition of Hedgehog signaling induces monocytic differentiation of HL-60 cells

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Running title: Cyclopamine induces monocytic differentiation

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Abstract

There is little evidence to demonstrate the importance of sonic hedgehog homolog (Shh) pathway to differentiation therapy in the treatment of hematological neoplasms. Here we characterize the changes in acute myelogenous leukemia (HL-60) cells after blocking the Shh pathway by an antagonist of Smoothened, cyclopamine. Cyclopamine induces apoptosis of HL-60 cells in a dose- and time-dependent manner with increased G0/G1 cycle fraction. Treatment with cyclopamine increases the expression of monocytic cell markers CD 11b and CD 14, but the expression of CD 13, CD 33 and CD 38 was unchanged. The monocytic differentiation of HL-60 cells induced by cyclopamine is also evidenced by an increase in Egr-1 expression. Importantly, cyclopamine down regulates the phosphorylation of Akt and ERK, but activates AMPK signaling. Further investigations should determine the clinical application of modulating the Shh pathway in the treatment of hematological malignancies.

Introduction

Hedgehog (Hh) proteins are a family of intercellular signaling molecules initially identified as polarization genes required for embryonic patterning in *Drosophila* [1]. Among the three mammalian homologs, sonic hedgehog (Shh) is most extensively studied. The Shh molecule is a transmembrane protein that mostly exhibits its function through cell-to-cell contact between adjacent cells expressing Patched (Ptc), a receptor protein of Shh.

Shh is known to play a critical role in normal cellular expansion and in the patterning of the early embryo of vertebrates and invertebrates; it is also implicated in human cancer [2]. It regulates a gamut of tissue development processes in skin, nervous system, foregut, lung, limbs, pituitary gland and pancreas [3]. In terms of tumorigenesis, *Ptc* has been described as a tumor suppressor gene; *Smo* and its downstream signaling *Gli* genes are oncogenic [4,5]. Medulloblastoma and basal cell carcinoma are two malignancies most associated with the hedgehog pathway [6,7], but Shh has also been implicated in small cell lung cancer and cancers of the stomach, esophagus, pancreas, biliary tract and prostate gland [8-11]. Recently, Yoshizaki A and colleagues reported an important role of the Hh pathway in myogenic differentiation and the malignant potential of human intestinal stromal tumors [12].

While the Hh protein family is certainly involved in the development and embryonic specification of nonhematopoietic tissues, the role of Hh function in hematopoietic tissue is less defined [13-15]. Some studies demonstrate a pivotal role for the Shh pathway in regulating the proliferation of primitive human hematopoietic cells [13], in early lymphoid cell differentiation [16-18], in erythrocyte proliferation and differentiation [19], and in cytokine production and promoting cell cycle progression in activated peripheral $CD4^+$ T lymphocytes [20,21]. Other studies suggest that the Shh pathway is not important in hematopoietic stem cell function [14,15]; of special interest is its role in hematological malignancies. Previously we showed via immunohistochemical staining that proteins of the Shh pathway are expressed variably in acute promyelocytic leukemia cells, acute myelogenous leukemia cells and multiple myeloma cells [22]. More direct evidence suggesting that this pathway is involved in hematological malignances gleans from studies in Bcr-Abl-positive leukemic stem cells [23,24], B-cell lymphoma and myeloma [25], and B-cell chronic lymphocytic leukemia [26]. By cross-talking with Wnt, Hox and Notch signaling, Shh emerged as playing a role in chronic myelogenous leukemia progression [27]. Furthermore, Kobune M and colleagues demonstrated how resistance to chemotherapy can be reduced by inhibiting the Shh pathway [28].

While evidence implicates a role of Shh pathway in hematopoiesis and in maintenance of hematological malignancy, little is known of its ability to differentiate leukemia cells. This study probes changes in HL-60 cells after modulating this pathway by cyclopamine, an antagonist of Smoothened protein.

Materials and methods

Cells

HL-60, an acute myelogenous leukemia cell line obtained from American Type Culture Collection (Manassas, VA), was cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and penicillin (100 U/mL)/streptomycin (100 μg/mL) (Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% CO_2.

Reagents

Cyclopamine was purchased from Sigma-Aldrich (C4116) and was dissolved in DMSO at a concentration of 20 μM as a stock solution for further use. Granulocyte

colony-stimulating factor was kindly provided by Chugai Pharmaceutical Co, Japan.

Apoptosis and cell cycle assay

Apoptosis was evaluated by dual staining with annexin V conjugated to flourescein isothiocyanate (FITC) and propidium iodide (PI). Cells (1×10^6) were stained by annexin V-FITC (BD Pharmingen, San Diego, CA) and PI (BD Pharmingen) according to the manufacturer's instructions, and analyzed by a Beckman-Coulter EPICS XL cytometer (Beckman-Coulter, Miami, FL). The cell cycle was assessed by propidium iodide staining and analyzed by flow cytometry.

Reverse transcription, polymerase chain reaction (PCR) and real-time PCR

Cells (5×10^6) were washed with PBS twice and mRNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA). The quantification and purification of mRNA was determined by absorbance in a spectrophotometer at 260 nm and 280 nm. The RNA was reverse transcribed into complementary DNA (cDNA) in a 20 μL volume (2 μg RNA, 1 μg oligo-dT, 1 μL of 10 mM dNTP, 1.5 μL of MMV reverse transcriptase, and proper buffer). For PCR reaction, the cDNA was amplified on the

Thermal Cycler (Infinigen Biotech Incorporation). The primers for PCR were followings: (a) *Shh* forward 5'-GAAAGCAGAGAACTCGGTGG-3', *Shh* reverse 5'-GGAAAGTGAGGAAGTCGCTG-3' (product 170 bp); (b) *Ptc* forward 5'-GTGGCTGAGAGCGAAGTTTC-3', *Ptc* reverse 5'-TTCCACCCACAGCTCCTC-3' (product 163 bp); (c) *Smo* forward 5'-CTGGTACGAGGACGTGGAGG-3', *Smo* reverse 5'-AGGGTGAAGAGCGTGCAGAG-3' (product 132 bp); (d) *Gli1* forward 5'-CCACGGGGAGCGGAAGGAG-3', *Gli1* reverse 5'-ACTGGCATTGCTGAAGGCTTTACTG-3' (product 255 bp); (e) *Gli2* forward 5'-GAAGTTCGTGGACTCCTACAATAATGC-3', *Gli2* reverse 5'-GACTCACTGCTCTGCTTGTTCTGG-3' (product 264 bp); (f) *G3PDH* forward 5'-ACCACAGTCCATGCCATCAC-3', *G3PDH* reverse 5'-TCCACCACCCTGTTGCTGTA-3' (product 451 bp). The products of PCR were separated in 2% agarose gel.

For the real-time PCR, the cDNA was compared for *Smo*, *Gli1* and *Gli2* expression using Real-Time PCR 2X Master Mix-SQGR-V2 (Protech Technology) in ABI PRISM™ 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The real-time PCR was performed by amplification protocol consisting of 1 cycle of 2 min at 50℃, 1 cycle of 10 min at 95℃, and 45 cycles of 15 sec at 95℃

and 1 min at 60℃.

Cell surface markers analysis

Cells (1×10^6) from each sample were washed with PBS twice and incubated with specific antibody. After 30 minutes, cells were washed with PBS once and were analyzed by a Beckman-Coulter EPICS XL cytometer (Beckman-Coulter, Miami, FL). The antibodies were purchased from the following companies: anti-CD 11b, anti-CD 13, anti-CD 14, anti-CD 33, anti-CD 38; anti-mouse IgG2a (BD Pharmingen).

Western blotting

Cell lysates were prepared by exposing cells to RIPA buffer (150 mM NaCl, 50 mM Tris PH 8.0, 1% NP40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate). Protease inhibitor (Sigma-Aldrich, Saint Louis, MO) and phosphatase inhibitor cocktail (Calbiochem, Gibbstown, NJ) were added to RIPA buffer before lysing the cells. Antibodies against various proteins were obtained from the following sources: p-Akt (Ser473), p-Akt (Thr308), Egr-1 (Santa Cruz

Biotechnology, Santa Cruz, CA); Akt, ERK1/2, p-ERK1/2 (Thr202Tyr204), p38 mitogen-activated protein kinase (p38 MAPK), p-p38 MAPK (Thr180Tyr182), AMP-activated protein kinase (AMPK), p-AMPK (Thr172), (Cell Signaling, Danvers, MA); β-actin (Sigma-Aldrich, Saint Louis, MO). The goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugates and goat anti-mouse IgG-HRP conjugates were purchased from PerkinElmer life Sciences, Inc. (Boston, MA).

Statistical analysis

Independent *t*-test was used to evaluate the change of ΔCt in real-time RT-PCR analysis, the change of cell cycle fraction and the change of surface marker expression after cyclopamine treatment compared with the control group. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using SPSS for Windows (SPSS, Inc., Chicago, IL)

Results

Expression of mRNA of Shh signaling pathway proteins in HL-60 cells

To validate existence of Shh signaling pathway in HL-60 cells, 2 μg of RNA extract from cell lysates were used for reverse transcription and then polymerase chain reaction for *Shh*, *Ptc*, *Smo* and *Gli1* genes [Figure 1(A)]. *G3PDH* gene served as a control. All four genes were confirmed to be present in HL-60 cells.

Cyclopamine down regulates the mRNA expression of Smo, Gli1 and Gli2 in HL-60 cells

To evaluate the effect of cyclopamine on Shh signaling pathway, RNA extract from HL-60 cells treated with cyclopamine at concentrations of 0, 5 and 20 μM for 6 h was used for reverse transcription and real-time PCR analysis [Figure 1(B)]. As shown, cyclopamine at 20 μM significantly down regulated the mRNA expression of *Smo*, *Gli1* and *Gli2*. *G3PDH* gene served as a control.

Cyclopamine mediates apoptotic death of HL-60 cells

HL-60 cells (2.5×10^5 cells/mL) were incubated with medium or cyclopamine at concentrations of 5 μM, 10 μM and 20 μM for 6 h, 12 h and 24 h. Cells were stained with annexin V/PI and analyzed by flow cytometry. Cyclopamine induced

apoptosis of HL-60 as evidenced by increased proportions of annexin V+ cells (figure 2). For HL-60 cells treated with 10 μM cyclopamine, the annexin V+ cells accounted for 5.1%, 7.6% and 11.1% at 6 h, 12 h and 24 h, respectively ($p = 0.073$, <0.001, 0.003 respectively compared with control group). For cells treated with 20 μM cyclopamine, the annexin V+ cells were 23.2%, 62.4% and 93.1% at 6 h, 12 h and 24 h, respectively (all three $p < 0.001$ compared with control group).

Cyclopamine increases G0/G1-phase fraction of HL-60 cells

HL-60 cells (2.5×10^5 cells/mL) were incubated with medium or cyclopamine at concentrations of 5 μM, 10 μM and 20 μM. At 6 h, 12 h and 24 h, cells were harvested, stained with propidium iodide and analyzed for cell cycle fractions by flow cytometry (Figure 3). At 6 h, there was no significant difference between the treated and control groups. After 12 h, cyclopamine at 5 μM and 10 μM concentrations increased the G0/G1-phase fraction of cells to 52.4 % and 54.7 % compared with 46.3 % in the control group ($p = 0.007$ and 0.001 respectively compared with control group). At 24 h, cyclopamine at concentrations of 5μM and 10 μM increased the G0/G1-phase fraction of cells to 50.6% and 52.5%, versus 46.0% in the control group ($p = 0.001$ and 0.002 respectivelycompared with control

group). Cells treated with 20 μM cyclopamine for 12 h or 24 h were not analyzed because the majority of cells underwent apoptosis similar to the previous viability study by annexin V/PI staining.

Cyclopamine induces monocytic phenotypic differentiation of HL-60 cells

HL-60 cells (2.5×10^5 cells/mL) were incubated with medium or cyclopamine at concentrations of 5, 10 and 20 μM for 2, 4 and 6 days. At each time point, cells were stained with anti-CD 11b, anti-CD 13, anti-CD 14, anti-CD 33, anti-CD 38, and mouse IgG2a and analyzed by flow cytometry [Figure 4(A)]. Although treatment with cyclopamine did not change the cell surface expression of CD 13, CD 33 and CD 38, the expression of CD 11b and CD 14 increased in a dose- and time-dependent manner. At day 4, the expression of CD 11b increased to 43%, 46% and 51% at concentrations of 5, 10 and 20 μM of cyclopamine compared with 34% in the control group ($p<0.001$ in all three comparisons). At day 6, the values were 55%, 56% and 58% compared with 38% in control group ($p<0.05$, $p<0.001$ and $p<0.05$, respectively). There was a significant increase of CD 11b expression after cyclopamine treatment, as well as a significant increment of CD 14 expression ($p=0.04$ and $p=0.12$ for cells treated with cyclopamine at a

concentrations of 10 μM and 20μM for 6 days, respectively).

Cyclopamine induces Egr-1 expression level in a dose-dependent manner

Egr-1 is a protein associated with the development of hematopoietic progenitor cells along the monocyte-macrophage lineage [29,30]. HL-60 cells (2.5×10^5 cells/mL) were incubated with medium or cyclopamine at concentrations of 10 μM or 20 μM for 48 and 72 h. Twenty μg of protein lysate were immunoblotted with anti-Egr-1 antibody. There was increasing expression of Egr-1 protein in a dose-dependent manner after cyclopamine treatment [Figure 4(B)].

Cyclopamine down regulates phosphorylation of Akt and ERK and increases AMPK phosphorylation

Akt and ERK pathways play an important role in cell proliferation, survival and differentiation. Some reports suggest that these pathways are involved in Shh signaling [31,32]. In addition, AMPK is associated with induction of Egr-1 expression [33]. Here, addition of cyclopamine reduced the phosporylation of Akt at Ser473 and, to a lesser extent, at Thr 308 (Figure 5). Cyclopamine also down

regulated phosphorylation of ERK in a dose-dependent manner. In contrast, cyclopamine induced increased phosphorylation of AMPK at Thr 172. The results suggested down-regulation of Akt and ERK phosphorylation being compatible with the antiproliferative effect of cyclopamine, and implied AMPK phosphorylation correlating with Egr-1 expression in monocytic differentiation of HL-60 cells.

Discussion

Little is known about the potential clinical value of blocking the Shh pathway in the differentiation therapy of hematological neoplasms. To define the role of this pathway in hematogical malignancies and provide insight into its antagonism of cell differentiation, we investigated the response of HL-60 cells (an acute myelogenous leukemia cell line) to cyclopamine treatment. Here we demonstrate that blocking the Shh pathway via cyclopamine causes a time- and dose-dependent apoptotic cell death, increases the proportion of cells in G0/G1-phase and induces monocytic differentiation in HL-60 cells. Cyclopamine also down regulates the mRNA expression of genes in Shh pathways.

The observed pro-differentiation effect, highlighted by an increase in Egr-1 expression, which is noteworthy because differentiation therapy has been used

successfully for treating hematological malignancies. A well-known example is the use of arsenic trioxide in the treatment of acute promyelocytic leukemia. By inducing differentiation, malignant cells lose abilities of rapid proliferation, self-renewal and resistance to drugs [34-36]. Cell biology studies have shown the dual effects of arsenic trioxide on acute promyelocytic leukemia cells -- differentiation and apoptosis induction [37]. The differentiation of leukemic cells induced by arsenic trioxide may involve the degradation of PML-RARs protein [37-39]. Recently, Beauchamp EM et al. and Kim J et al. have shown arsenic trioxide inhibit tumor growth by interacting with the Gli family proteins [40,41]. In our study, inhibiting Shh pathway by cyclopamine reduced HL-60 cell growth and induced monocytic differentiation. Because cyclopamine antagonizes Smoothened which is upstream to Gli transcriptional factor, it is speculated that arsenic trioxide induces cell differentiation by interaction with Gli family. Another appealing subject is to investigate the combinatorial use of arsenic trioxide and inhibitors of Shh pathway.

The ability of cyclopamine to cause myeloid blasts to differentiate into cells with monocytic phenotype is interesting when compared with the finding of Detmer and colleagues [19]. When normal marrow mononuclear cells were grown in the presence of 10 μM cyclopamine, development of colonies of

granulocytic/monocytic lineage was unaffected in terms of both number and morphology. However, the number of erythroid colonies was significantly reduced [19]. Compared with our finding, it appears that inhibition of Shh pathway by cyclopamine has a different effect on cell differentiation in different cell lineages. The inherent nature of the respective cell types and their sensitivity to external factors may play a role in their differing responses to cyclopamine. Additionally, the normal marrow mononuclear cells used by Detmer may have cellular biological responses different from the HL-60 cells.

Some studies supported the role of the Shh pathway in maintaining the malignant potential, and inhibition of the Shh pathway has been shown to cause cell apoptosis in some hematopoietic malignancies. Dierks C, et al. found that Shh pathway inhibition induced apoptosis in stroma-dependent lymphoma cells [25], and Kobune M et al. showed that inhibition of Shh signaling with cyclopamine induced apoptosis of $CD34^+$ leukemic cells [28]. Cyclopamine provably inhibits bone marrow stromal cell-induced survival of B-CLL cells by inducing apoptosis [26]. The present study demonstrates cyclopamine down-regulating phosphorylation of Akt and ERK1/2 which are involved in the antiproliferative activity as described in previous reports [31, 32]. In addition, in agreement with data in previous reports [33], this study suggests that AMPK activation is involved

in Egr-1 expression and in the pro-differentiation activity of cyclopamine.

In conclusion, inhibiting the Shh pathway by cyclopamine leads to apoptotic cell death, increases the proportion of cells in the G0/G1 cycle fraction, and induces monocytic differentiation of HL-60 cells. The antiproliferative and differentiation-inducing activities of cyclopamine correlate with decreasing Akt and ERK1/2 phosphorylation and increasing AMPK phosphorylation. This result implicates the Shh pathway in the maintenance of prototypic characteristic of HL-60 cells. Most notably, the prognostic and predictive value of Shh pathway expression in hematological malignancies merits further investigations, and the clinical application of modulating the Shh pathway to treat hematological malignancies remains to be clarified.

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Declaration of Interests: The authors have no conflict of interest to disclose.

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Figure Legend

Figure 1. Sonic hedgehog pathway gene expression in HL-60 cells and the response to cyclopamine treatment. (A) Reverse transcription and polymerase chain reaction (RT-PCR) of sonic hedgehog pathway genes in HL-60 cells. G3PDH was used as a control. The data shown here represent three independent experiments with similar results. (B) Real-time RT-PCR analysis of HL-60 cells treated with cyclopamine for 6 h. Cyclopamine at 20 μM down regulated the mRNA expression of Smo, Gli1 and Gli2 as evidenced by higher ΔCt values (n=3). ∗ denotes $p < 0.05$ and # denotes $p < 0.001$ when compared with control group.

Figure 2. Cyclopamine mediates apoptotic death of HL-60 cells. Cells (2.5×10^5 cells/mL) were incubated with medium or cyclopamine at concentrations of 5 μM, 10 μM and 20 μM for 6 h, 12 h and 24 h. Cells were stained with annexin V/propidium iodide and analyzed by flow cytometry. The value represents the average of percentage of cells in each quadrant (n=3).

Figure 3. Cell cycle analysis of HL-60 cells. HL-60 cells (2.5×10^5 cells/mL) were incubated with medium or cyclopamine at concentrations of 5 μM, 10 μM and 20 μM. At 6 h, 12 h and 24 h, cells were harvested, stained with propidium iodide and

analyzed for cell cycle fractions by flow cytometry. The number in each bar segment represents the relative percentage of cells in each cell cycle phase (n=3). Cells treated with 20 μM cyclopamine for 12 h or 24 h were not analyzed because majority of cells underwent apoptosis. ∗ denotes $p < 0.05$ when comparing the G0/G1 fraction in treated cells with that in control group.

Figure 4. Cell surface marker expression and Egr-1 protein blot of HL-60 cells. (A) Surface marker was expressed as percentage of cells expressing the indicated markers. Cells were incubated with medium or cyclopamine for 2, 4 and 6 days. Mouse IgG2a was used as a control (n=3). ∗ denotes $p < 0.05$, # denotes $p < 0.001$ when compared with control group. (B) Twenty μg of total cell lysate were immunoblotted with indicated antibodies.

Figure 5. Western blotting analysis of HL-60 cells treated with cyclopamine. Cells (2.5×10^5 cells/mL) were incubated with medium or cyclopamine in concentrations of 10 μM and 20 μM for 48 h.

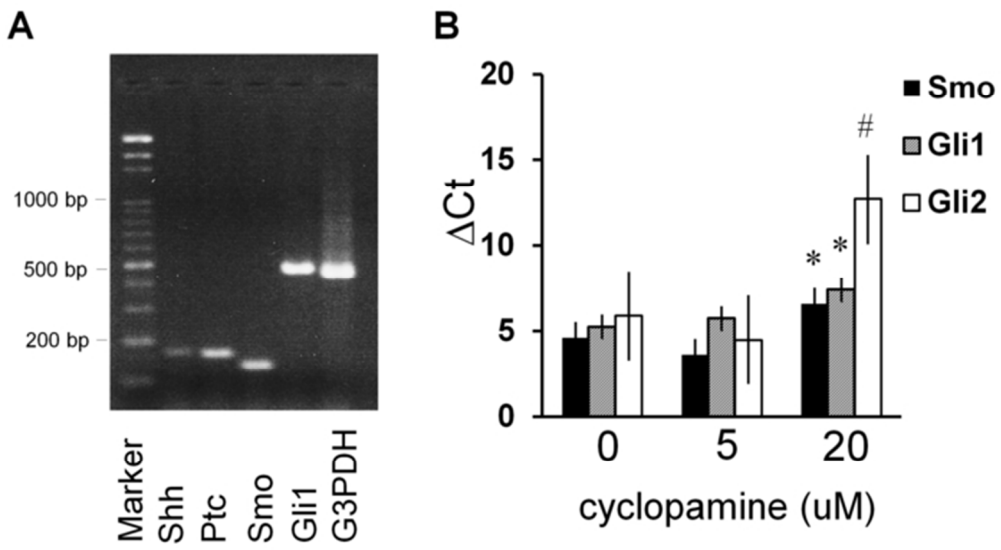

Fig 1
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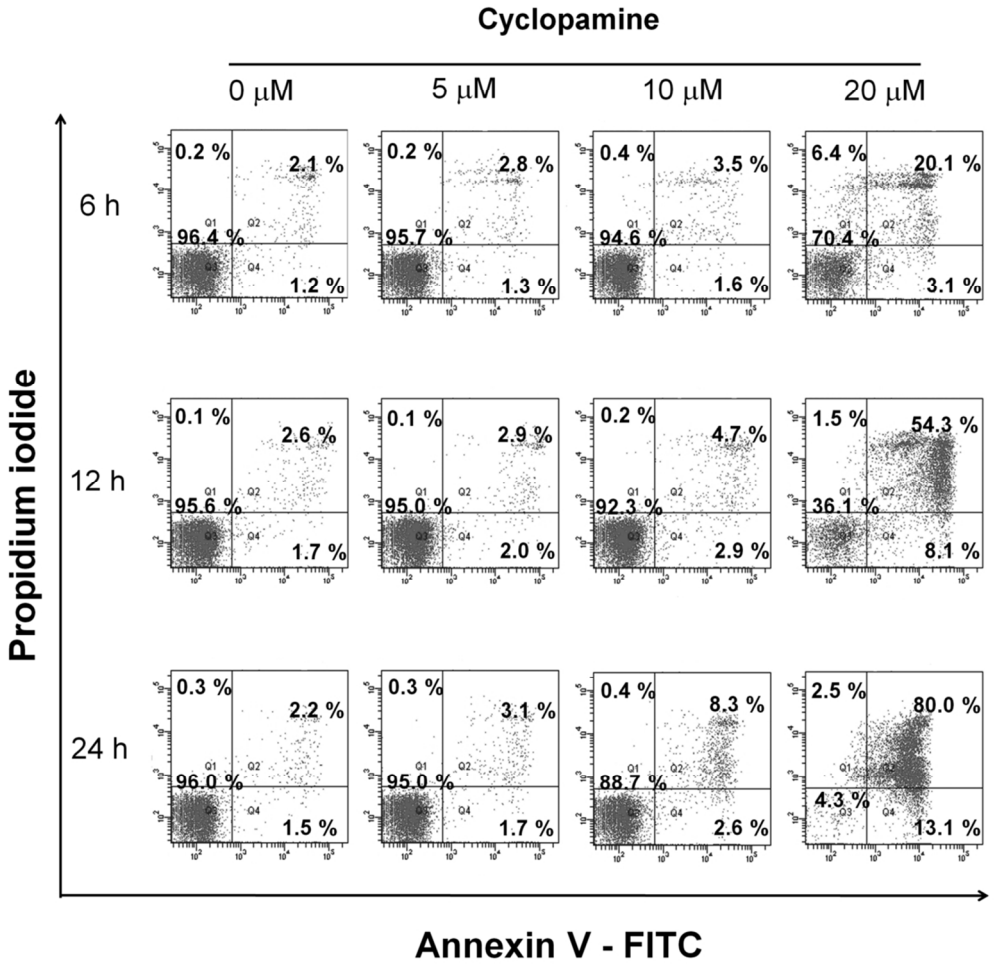

Fig 2
101x98mm (300 x 300 DPI)

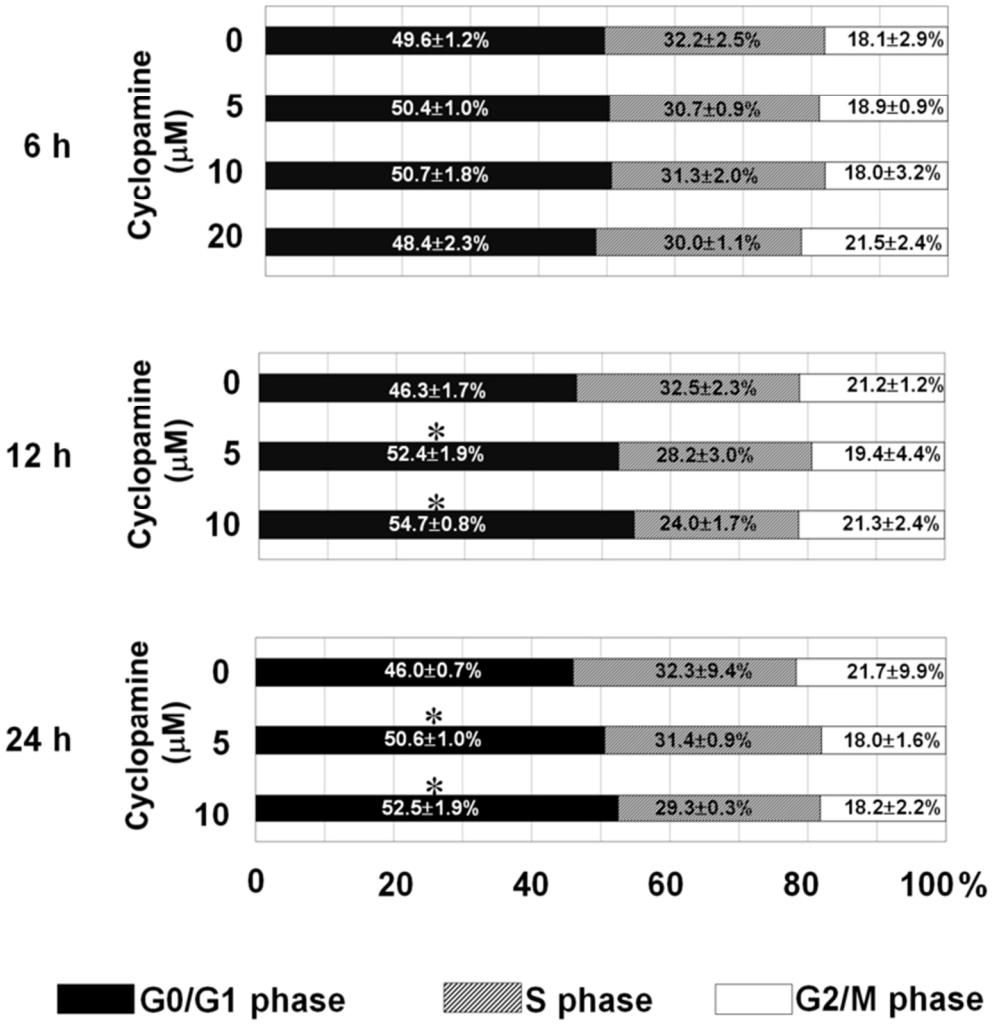

Fig 3
68x70mm (300 x 300 DPI)

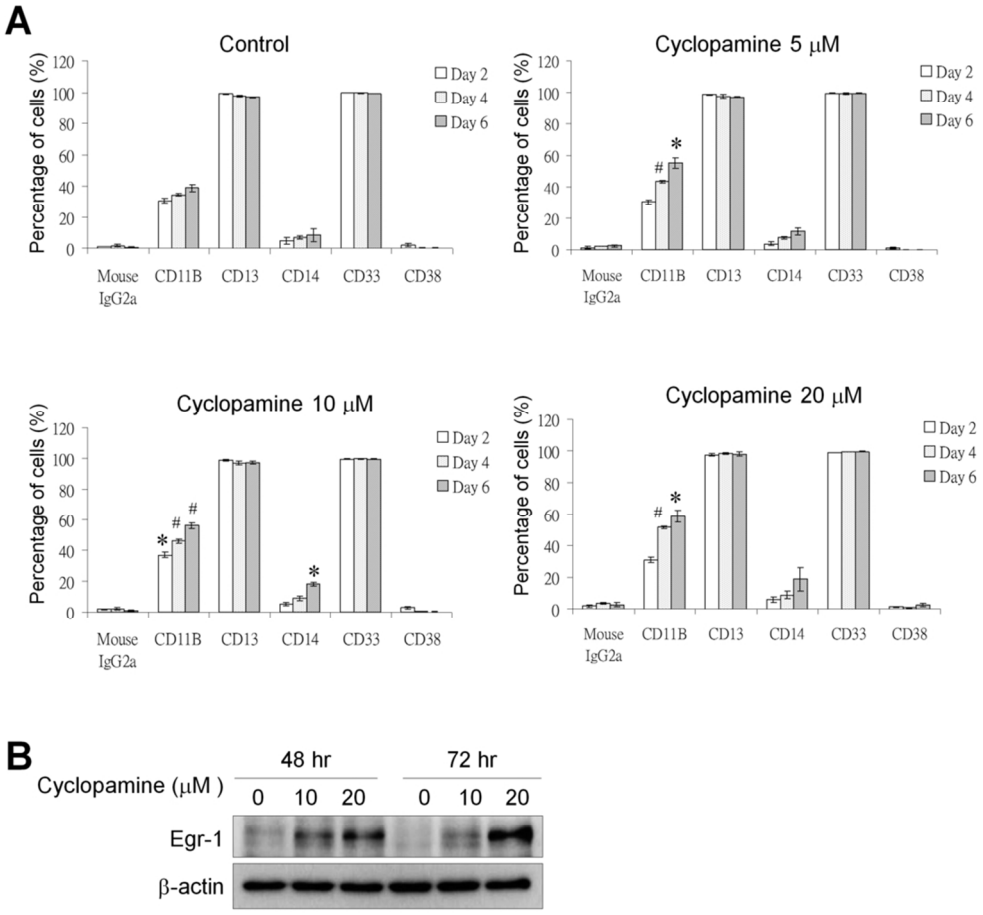

Fig 4
94x87mm (300 x 300 DPI)

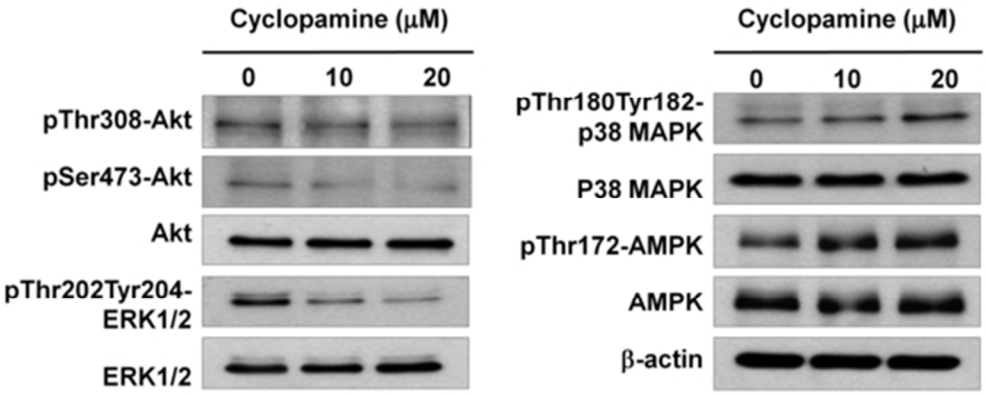

Fig 5
49x19mm (300 x 300 DPI)