



Different effects of phosphatidylinositol 3-kinase inhibitor LY294002 and Akt inhibitor SH-5 on cell cycle progression in synchronized HL-60 leukemia cells

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Abbreviations:

ATRA – all-*trans*-retinoic acid
DMSO – dimethyl sulfoxide
FBS – fetal bovine serum
PH domain – pleckstrin homology domain
PI – propidium iodide
PIA – phosphatidylinositol ether lipid analogue
PI3K – phosphatidylinositol 3-kinase
PKB/Akt – protein kinase B/Akt

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Abstract

Background and Purpose: Pharmacological inhibitors of phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway have been proposed in treatment of leukemia. Our previous studies demonstrated that PI3K inhibitor LY294002 and Akt inhibitor SH-5 reduced the number of viable HL-60 leukemia cells, but exerted different effects on their differentiation. PI3K inhibitor LY294002 induced an arrest of HL-60 cells in G₀/G₁ and G₂/M phases of the cell cycle, but the effects of Akt inhibitor on cell cycle progression have not been investigated. The present study was undertaken in order to compare the effects of LY294002 and SH-5 on cell cycle progression in a model of aphidicolin and nocodazole-synchronized HL-60 cells.

Materials and Methods: HL-60 cells were arrested at G₁/S phase by aphidicolin, or G₂/M phase by nocodazole, washed and allowed to progress synchronously through the cell cycle. PI3K inhibitor LY294002 (10 μM) and Akt inhibitor SH-5 (20 μM) were added to medium after release from the block. DNA content was determined by propidium iodide staining and flow cytometry.

Results: In aphidicolin-synchronized HL-60 cells, LY294002 induced a delay in progression through S and G₂/M phases, while Akt inhibitor had no significant effects. No statistically significant effects were observed in LY294002 or SH-5-treated cells at 1–5 h after release from nocodazole block.

Conclusion: PI3K inhibitor LY294002 and Akt inhibitor SH-5, applied at doses that effectively inhibit Akt-activity, have different effects on cell cycle progression in aphidicolin-synchronized HL-60 cells, suggesting that LY294002-induced delay in S and G₂/M phase is probably not due to the specific inhibition of Akt-activity.

INTRODUCTION

Phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway regulates many crucial cellular processes, including proliferation, growth, metabolism, cell motility, survival and hematopoiesis. PI3K family of lipid kinases is classified into three main classes (PI3KI-III); the class I is present in different cell types and it is responsible for phosphorylation of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) into phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃). Upon formation, PtdIns(3,4,5)P₃ induces recruitment of protein kinase B (PKB)/Akt through the phosphoinositide-binding pleckstrin homology (PH) domain, followed by conformational change, phosphorylation and ac-

tivation of Akt. Once fully activated, Akt phosphorylates downstream targets that are involved in regulation of cell growth, proliferation, differentiation and survival (1–3).

As the upregulation of PI3K/Akt pathway has been found to be a common hallmark of many human tumors, including acute leukemia (2, 3), it has been proposed that the selective PI3K/Akt pharmacological inhibitors might be used in leukemia therapy in combination with cytotoxic drugs, radiation or all-*trans*-retinoic acid (ATRA) (4). Two potent and cell-permeable PI3K-inhibitors have been widely used to study the role of PI3K *in vitro*; wortmannin and LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (5, 6), and several studies have demonstrated the antiproliferative effects of LY294002 on leukemia cell lines (7–9). Novel strategies for selective inhibition of Akt include the development of phosphatidylinositol ether lipid analogues (PIA) that cannot be phosphorylated by PI3K on the 3-position of the *myo*-inositol ring, but bind to the PH domain of Akt, thus acting as competitive inhibitors for Akt activation (10, 11). Our previous study demonstrated the beneficial effects of commercially available PIAs, Akt inhibitor I (1L-6-hydroxymethyl-chiro-inositol 2(R)-2-*O*-methyl-3-*O*-octadecylcarbonate) and SH-5/Akt inhibitor II (D-3-deoxy-2-*O*-methyl-*myo*-inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate]) on HL-60 leukemia cells (9). The presence of Akt inhibitors reduced the number of both control and ATRA-treated HL-60 cells, while having no inhibitory effects on ATRA-mediated differentiation. In contrast, antiproliferative effects of LY294002 were found to be associated with a reduction in the percentage of cells with differentiated phenotype. As the effects of lipid-based inhibitors of Akt on differentiation of leukemia cells differed from the effects of both LY294002 and down-modulation of Akt by siRNA in the same cell line, it was suggested that the effects could be attributed to some other target molecules that are influenced by the pharmacological inhibitor in HL-60 cell line, but the mechanisms responsible for differences in the effects of Akt inhibitor and LY294002 were not investigated.

Although LY294002 is used as an inhibitor of PI3K, it also inhibits several other enzymes involved in cell cycle regulation, including ATM/ATR (12) and DNA-PK (13). Our previous studies demonstrated that antiproliferative effects of LY294002 on HL-60 cells were associated with an arrest of cells in G₀/G₁ and G₂/M-phases of the cell cycle, but the effects of Akt inhibitors on HL-60 cell cycle progression were not investigated (7). The present study was undertaken in order to compare the effects of LY294002 and Akt inhibitor II (SH-5) on cell cycle progression in a well-described model of aphidicolin and nocodazole-synchronized HL-60 cells.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS), penicillin-streptomycin and L-glutamine were obtained from Gibco/Invitrogen

(Grand Island, NY, USA). Propidium iodide (PI), RNase A, Tris, Igepal, dimethyl sulfoxide (DMSO), aphidicolin and nocodazole were purchased from Sigma (St Louis, MO, USA). Aphidicolin and nocodazole were dissolved in 100% DMSO to a stock concentration of 10 mg/mL and 200 µg/mL, respectively.

PI3K inhibitor LY294002 and Akt inhibitor II (SH-5) were obtained from Calbiochem (San Diego, CA, USA). The inhibitors were dissolved in 100% DMSO to a stock concentration of 25 mM (LY294002) and 20 mM (Akt inhibitor II, SH-5). Other chemicals were of analytical grade.

Cell culture and cell cycle synchronization

HL-60 human promyelocytic leukemia cell line (ECCACC No. 88112501) was purchased from the European Collection of Animal Cell Cultures, PHLS, Porton, Salisbury, UK. The cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 µg/mL streptomycin and 100 units/mL penicillin at 37°C in a humidified 5% CO₂ environment. The cells were passaged twice a week to maintain exponential growth. The number of viable cells was determined by using a Bürker-Türk hemocytometer and trypan blue staining.

In experiments, exponentially growing cells were harvested, washed and resuspended in FBS-containing RPMI medium at the initial cell concentration of 0,25–0,3 × 10⁶/mL. To induce G₁/S arrest, cells were incubated in the presence of aphidicolin (2 µg/mL) for 18 h, as previously described (14–16). After the incubation, cells were released from the block by washing four times with FBS-free RPMI medium. To induce an arrest at metaphase, cells were incubated in the presence of nocodazole (200 ng/mL) for 24 h, as previously described (16, 17). After the incubation, cells were released from the nocodazole block (time 0) by washing four times in fresh medium, as described above.

After the block, cells were resuspended in fresh medium containing 10% FBS and incubated further in the presence of one of the following agents: Akt inhibitor SH-5 (20 µM), PI3K inhibitor LY294002 (10 µM) or DMSO vehicle alone (control). At time points indicated, cells were harvested, stained with propidium iodide and analyzed by flow cytometry.

Flow cytometric analysis

The analysis was performed as previously described (15–17). Briefly, cells were collected, washed with 2 mL of ice-cold PBS and stained directly with 300 µL of propidium iodide solution (10 mM Tris, pH 8.0, 10 mM NaCl, 10 µg/mL RNase, 50 µg/mL PI, 0,1% Igepal) for 30 min at 4°C. DNA content was determined using FACSCalibur system and Cell Quest software (Becton Dickinson). Acquired events were gated for exclusion of debris and cell aggregates. DNA analysis was performed on 15,000 gated events for each sample. ModFit software

(Becton Dickinson) was used in order to determine the percentage of cells in the particular phase of cell cycle.

Statistical analysis

The data are presented as means \pm S.E.M and evaluated with Student's *t*-test for unpaired samples. $P < 0,05$ was considered as statistically significant.

RESULTS

Incubation of HL-60 cells in the presence of aphidicolin, an inhibitor of DNA polymerase, causes the majority of cells to arrest at the G₁/S boundary. After release from the block, cells synchronously enter into S phase and continue to cycle (14–16). To test for the possible effects of PI3K and Akt inhibitors on the cell cycle progression through the S phase, HL-60 cells were first treated with aphidicolin for 18 h, and then released from the block by washing and resuspending in fresh medium containing one of the inhibitors or DMSO solvent alone. PI3K inhibitor LY294002 and Akt inhibitor SH-5 were applied in doses that were previously shown to be effective in blocking Akt activation in HL-60 cells (9). At time indicated, the cells were harvested, stained with PI, and DNA analysis was performed by FACS. As shown on Figure 1, control cells that were released from G₁/S-block (time 0) synchronously progressed through the S phase and reached G₂/M phase at 8 h. The presence of Akt inhibitor II (20 μ M) had no noticeable effects on the cell cycle progression at the indicated time points. However, a delay in cell cycle progression was detectable in cells treated with PI3K inhibitor LY294002 (10 μ M), being most prominent at 6 h after release from aphidicolin block, which is consistent with the progression delay through the S phase (Figure 1).

To further investigate the possible effects of PI3K and Akt inhibitors on progression through G₂/M phase, aphidicolin-synchronized cells were analyzed at 10 to 16 h after release from the block. As shown in Figure 2a and 2b, the majority of control cells progressed through the G₂/M and returned to G₀/G₁ phase after 10–12 h. Again, the cell cycle distribution of HL-60 cells incubated in the presence of Akt inhibitor did not differ from the distribution of control cells. In contrast, treatment with LY294002 induced a significant increase in the proportion of cells in G₂/M phase at 10 h after the block (Figure 2b), which was associated with a decrease in the population of cells in G₀/G₁ phase, and an increase in the fraction of cells in sub-G₁ (Figure 2a). However, PI3K inhibitor-treated cells progressed eventually through the G₂/M phase, as detected at 12 and 14 h after the block. A delay in cell cycle progression was apparent again at 16 h after the block; while control cells had completed the cycle, passed through the G₁/S checkpoint, and initiated DNA replication, significant proportion of LY294002-treated cells had been delayed in G₀/G₁, causing a decrease in the percentage of cells in S phase, as measured by ModFit (Figure 2b).

To further dissect the timing of LY294002-induced delay in G₂/M phase progression in aphidicolin-synchronized HL-60 cells, cells were incubated in the presence of nocodazole, a microtubule inhibitor that arrests the cells in metaphase. After release from the block, cells complete mitosis and go synchronously through G₁ phase, thus allowing a study of the events associated with M/G₁ transition and early G₁ phase (16). As shown in Figure 3a, majority of HL-60 cells were arrested in G₂/M phase after incubation in the presence of 200 ng/mL of nocodazole for 24 h (time 0). After removal of nocodazole by washing and reseeding of cells in the fresh me-

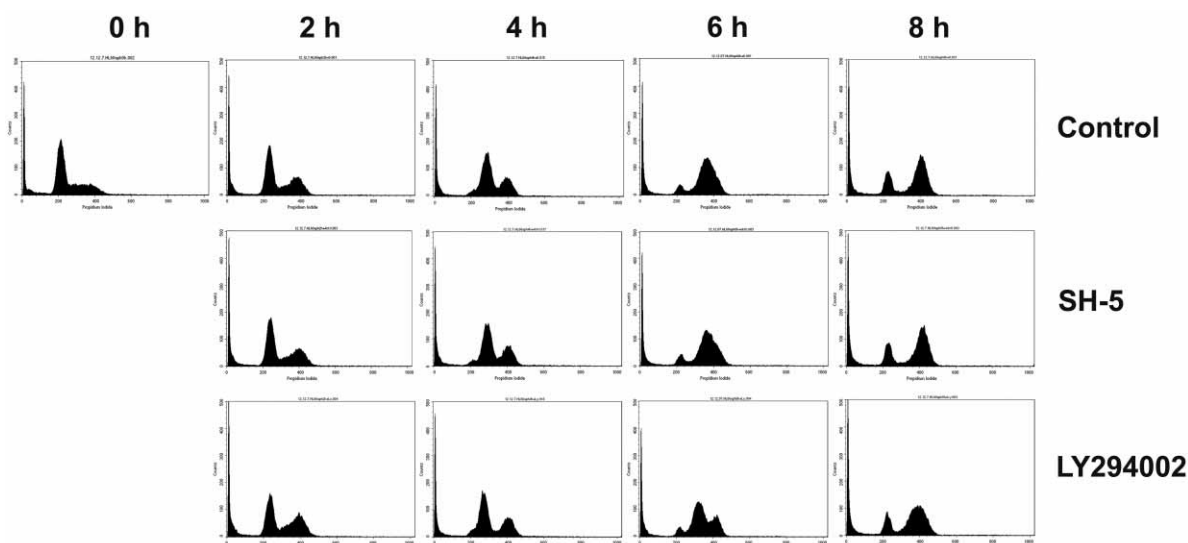


Figure 1. The effects of PI3K inhibitor LY294002 and Akt inhibitor SH-5 on cell cycle progression of aphidicolin-synchronized HL-60 cells through the S phase. Cells were incubated in the presence of aphidicolin (2 μ g/mL) for 18 h. After release from aphidicolin block by washing and resuspending the cells into fresh FBS-containing medium, cells were incubated with DMSO solvent alone (control), LY294002 (10 μ M) or SH-5 (20 μ M), respectively. Cells were harvested at 0–8 h after release from the aphidicolin arrest, stained with PI solution and DNA content was determined by FACS.

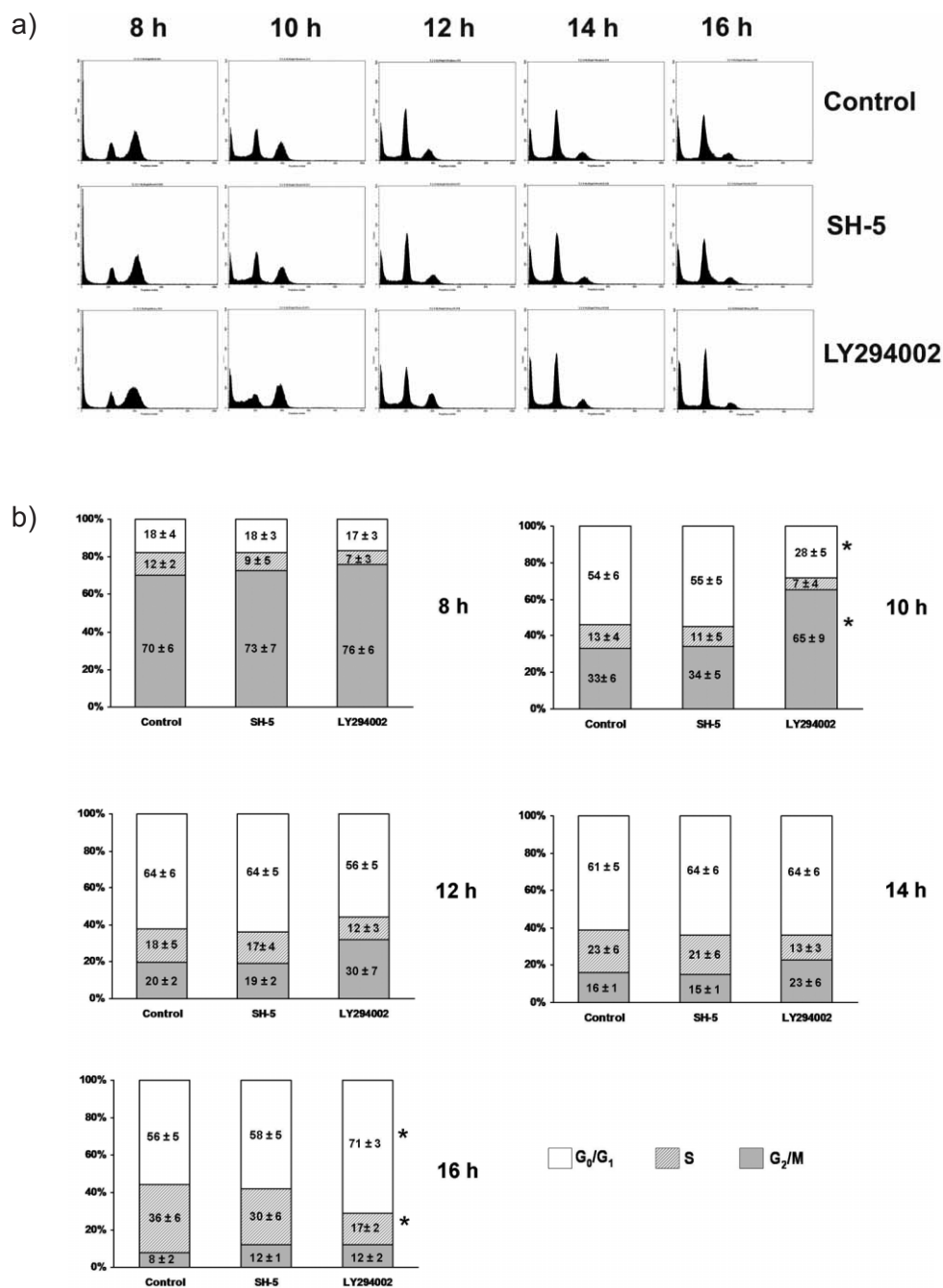


Figure 2. The effects of PI3K inhibitor LY294002 and Akt inhibitor SH-5 on cell cycle progression of aphidicolin-synchronized HL-60 cells through the G₂/M phase. After release from the aphidicolin arrest, cells were incubated with DMSO solvent alone (control), LY294002 (10 μM) or SH-5 (20 μM) for 8–16 h. Cells were stained with PI solution and DNA content was determined by flow cytometry. (a) Representative histograms and (b) ModFit analysis of cell cycle distribution of aphidicolin-synchronized HL-60 cells harvested at 8–16 h after release from G₁/S arrest. The values represent the mean ± SEM from at least three different experiments. * P < 0,05 in comparison to control

dium, the percentage of cells in G₂/M phase progressively declined, followed by an increase in the proportion of G₀/G₁ cells, which indicated the completion of mitosis and progression through early G₁ phase (Figure 3a and b). No statistically significant differences have been observed in cell cycle distribution of LY294002 or SH-5-treated cells in comparison to control cells at 1–5 h after release from nocodazole block (Figure 3b).

DISCUSSION

An extensive effort has been invested in developing methods to selectively and efficiently inhibit components of PI3K/Akt signaling pathways. In 2000, Kozikowski *et al.* designed phosphatidylinositol lipid analogues that target the PH domain of Akt and potently inhibit Akt in several cell lines, including leukemia cells (10, 18). Results from our previous study confirmed growth-inhibi-

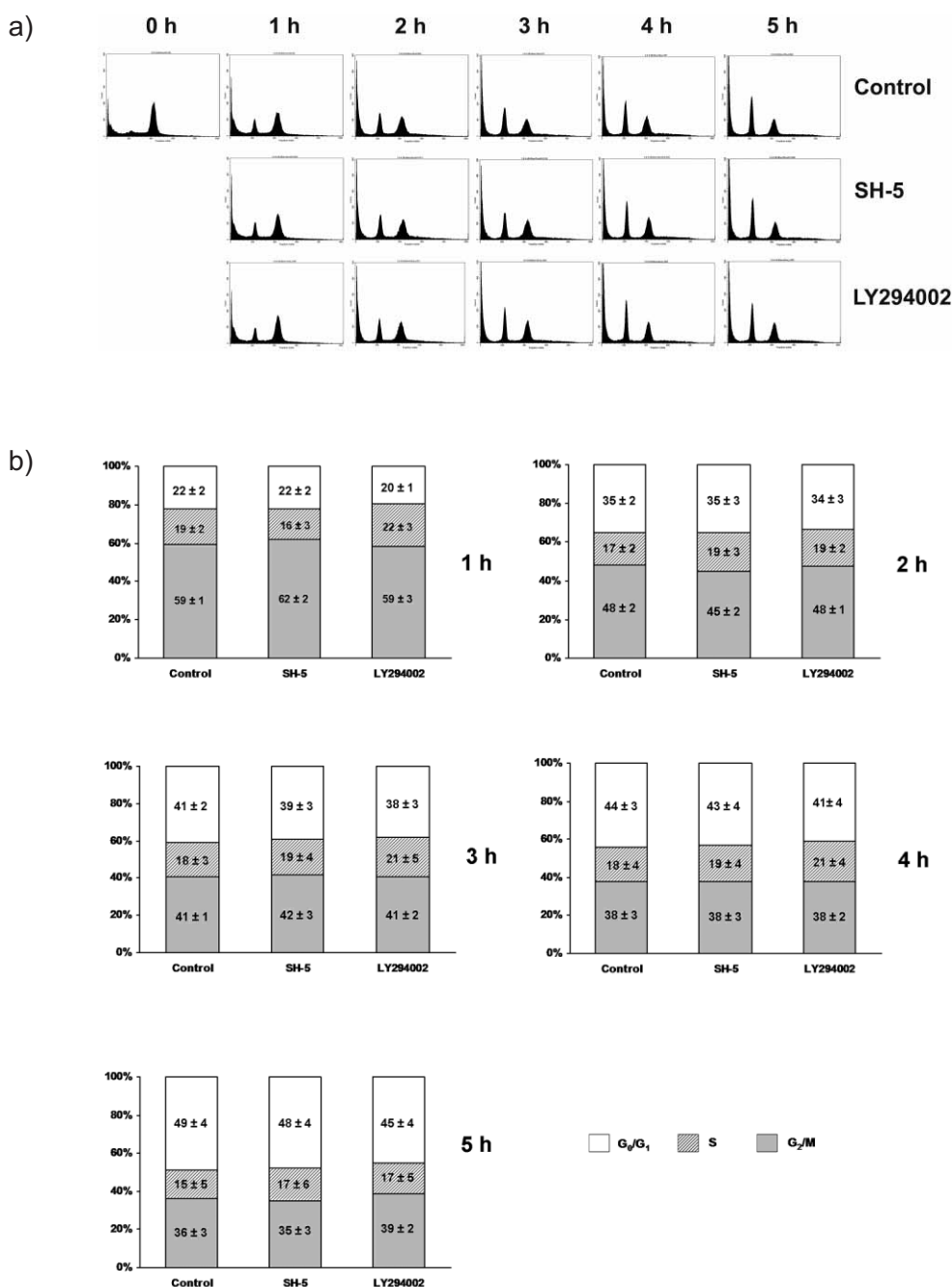


Figure 3. The effects of PI3K inhibitor LY294002 and Akt inhibitor SH-5 on cell cycle progression of nocodazole-synchronized HL-60 cells. Cells were incubated in the presence of nocodazole (200 ng/mL) for 24 h. After release from the arrest, cells were incubated with DMSO solvent alone (control), LY294002 (10 μM) or SH-5 (20 μM) for 1–5 h. DNA content was determined by PI staining and FACS analysis. (a) Representative histograms and (b) Mod Fit analysis of cell cycle distribution of nocodazole-synchronized HL-60 cells harvested at 0–5 h after release from metaphase arrest. The values represent the mean ± SEM from at least three different experiments. * *P* < 0,05 in comparison to control

tory properties of novel PIA-based inhibitors and showed a beneficial effect of SH-5 on differentiation of control and ATRA-differentiated HL-60 cells. When applied at doses that inhibit Akt activity, LY294002 and Akt inhibitors exerted the similar growth-inhibitory effects, but differentiative properties were observed only in PIAs-treated HL-60 cells (9). Since then, several observations suggested that PIAs might have other targets in addition

to Akt, and that identifying »off target« effects could expand possible clinical applications or help to predict drug-mediated toxicities (19, 20). In doses that inhibit Akt, SH-5 and other PIAs have been reported to activate p38αMAPK (19) and AMP-activated protein kinase (20), the effects that are missing in LY294002-treated cells. A recent study, aimed to characterize Akt-independent effects of the synthetic Akt inhibitors using transcriptomic

profiling and signaling pathway perturbations, demonstrated that, among other changes, SH-5 and SH-6 down-regulate genes associated with the mitotic cell cycle (21).

A decrease in the number of viable HL-60 cells that was observed 24–48 h after addition of SH-5 or LY294002 could reflect a lower rate of cell cycle progression and/or increased cell death (9). Our previous study was performed in asynchronous population of HL-60 cells in which the majority of cells were in G₁-phase, so that potential effects that inhibitors might have in the later stages of the cell cycle might be concealed by the well-described role that PI3K/Akt inhibitors have in regulating progression through G₁ and into S-phase (22, 23). Therefore, the present study was performed in synchronized HL-60 cells and data obtained showed that the presence of LY294002 caused a significant delay in progression of aphidicolin-synchronized cells through multiple points, including S and G₂/M phase. The similar results have been obtained in double thymidine-blocked HeLa cells in which the addition of LY294002 (25 μM) immediately or 5 h after release induced a delay in S and G₂ phase, respectively (24). In synchronized NIH3T3 cells, LY294002 caused a delay in S phase exit and G₂/M entry, which was associated with a decrease in the level of cyclin B1 (25). In addition, a delay in G₂/M transition and an increase in sub-G₁ fraction in our model of LY294002-treated cells is quite similar to the effects of 30 μM LY294002 added to synchronized HeLa cells 5 h after the block; about 20% of HeLa cells died by apoptosis while passing through the G₂/M phase (26). Results from the present study also suggest that the effects of LY294002 on G₂/M phase is probably due to the effect on the cell cycle progression before cells reach metaphase-anaphase transition, as we observed no significant effects of the presence of PI3K inhibitor on the proportion of cells entering G₁ phase 1–5 h after release from the nocodazole block. These results are similar to those obtained in choroidal melanoma cells synchronized by the presence of nocodazole; the presence of 20 μM LY294002 had no significant effects on the cell cycle distribution early (2 h) after nocodazole release, but caused a well-described G₁ arrest 8–20 h after release from the block (27).

Previous reports concerning the role of Akt in progression through the G₂/M phase are contradictory. First report demonstrated that fibroblasts expressing constitutively active PI3K/Akt forms accumulate in G₂/M due to defective cytokinesis and delayed transition from M to G₁ (28). Later studies reported that activated Akt induced a promotion of mitosis after DNA damage (29, 30). In addition, suppressing Akt signaling with dominant-negative Akt was found to repress mitotic entry (25). Even the time-course of Akt activation during the cell cycle was reported to differ, depending on cell type, as the activity of Akt was found to increase in the late G₂/early M phase in synchronized HeLa and MDCK cells, while no increase was recorded in NIH3T3 cells (26). However, other groups reported an increase in Akt activity to occur during G₁/S transition and M phase entry in both U2OS and NIH3T3 cells (31). On the other hand, an increase in the

activity of Akt was found to occur at M/G₁ transition in PC12 cells (32).

Whatever is the role of Akt in progression through the mitosis, the results of our study show that the presence of Akt inhibitor SH-5, at a dose that was previously shown to inhibit an increase in the activity of ATRA-treated HL-60 cells as effectively as 10 μM LY294002 (9), has no effects on the progression of the cells well after restriction point in G₁ phase. Differences in the effects of LY294002 and SH-5 on the progression of the cells through S and G₂/M phase of the cycle may be explained by the possibility that Akt was not the only target of LY294002 that positively regulates entry into mitosis, and previous studies showing that inhibitory effects of LY294002 were only partially reversed in cells transfected by constitutively active Akt support the hypothesis (24). Of note is that LY294002 inhibits not only a wide group of target molecules that are known to be involved in regulation of the cell cycle, but also blocks the activity of other PI3Ks that are not related to Akt activity (5). One of the possible targets includes class II PI3K-C2β that we have previously shown to be activated in aphidicolin-synchronized cells 8 h after release from the block; the enzyme activity produced only phosphatidylinositol-3-phosphate (PtdIns(3)P), which did not activate Akt, but the activation of PI3K-C2β was completely inhibited by LY294002 (15).

In conclusion, data from the present study show that PI3K inhibitor LY294002 and Akt inhibitor SH-5, applied at doses that effectively inhibit Akt activity, have different effects on cell cycle progression in aphidicolin-synchronized HL-60 cells, suggesting that LY294002-induced delay in S and G₂/M phase is probably not due to the specific inhibition of Akt activity.

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