Inhibition of CK2α and PI3K/Akt Synergistically Induces Apoptosis of CD34+CD38− Leukaemia Cells while Sparing Haematopoietic Stem Cells

JUNE-WON CHEONG1, YOO HONG MIN1,2, JU IN EOM3, SOO JEONG KIM1, HOI KYUNG JEUNG3 and JIN SEOK KIM1

1Department of Internal Medicine, 2Center for Chronic Metabolic Disease Research, 3Medical Research Center, Yonsei University College of Medicine, Seoul 120-752, Korea

Abstract. Background/Aim: The CD34+CD38− leukaemia cell population contains leukaemia stem cells (LSCs) responsible for treatment failure in acute myeloid leukaemia (AML) and, thus, novel therapies are required to eradicate LSCs without harming healthy haematopoietic stem cells (HSC). Materials and Methods: The present study evaluated the effects of co-treatment with LY294002 (a PI3K/Akt inhibitor) and apigenin (a CK2 inhibitor) (LY/Api) at subtoxic concentrations on leukaemia cell lines and primary AML cells. Results: LY/Api synergistically induced apoptosis in leukaemia cells, especially CD34+CD38− leukaemia cells. However, these effects were negligible in HSCs. LY/Api-induced apoptosis was accompanied by activation of caspase cascades and disruption of mitochondrial membrane potential. Caspase inhibitor or Akt overexpression abrogated this synergistic induction in apoptosis by LY/Api. LY/Api also led to remarkable down-regulation of anti-apoptotic proteins including Bcl-xL and NF-κB in CD34+CD38− leukaemia cells, but not in healthy hematopoietic stem cells. Conclusion: Inhibition of both CK2 and PI3K/Akt pathways may be a promising LSCs-targeted therapeutic strategy for AML.

Acute myeloid leukaemia (AML) arises from a rare subpopulation of leukaemia stem cells (LSCs), originating from malignant transformation of hematopoietic stem cells (HSCs) or progenitors (1-5). LSCs, presented in CD34+CD38− cell population, are typically found as quiescent state and may overexpress the multidrug efflux pump (6, 7). Thus, standard chemotherapy that preferentially eradicates actively cycling cells (8) is not sufficiently toxic to LSCs (9) and the identification of novel therapeutics to eradicate LSCs without harming HSCs is required.

Several recent studies demonstrated that the PI3K/Akt axis is constitutively activated in AML cells and associated with a lower survival rate (10, 11). These results suggested that the aberrant activation of the PI3K/Akt may be one of the critical mechanisms underlying drug resistance in AML. In addition, dysregulation of CK2 (formerly casein kinase II) is also critically involved in cell growth promotion and inhibition of apoptosis (12-14). It was previously reported that elevated levels of the catalytic subunit of CK2, CK2α, are frequently observed in primary AML cells and are highly associated with poor prognosis in AML patients with normal karyotype (15). Furthermore, some studies documented that CK2α may act in concert with Akt for promoting proliferation and protecting cells from apoptosis (16, 17).

The present study evaluated the effect and mechanism of co-treatment with the CK2 inhibitor, apigenin, and the PI3K/Akt inhibitor, LY294002, on leukaemia cell lines and primary AML cells, especially the CD34+CD38− leukaemia cell population containing LSCs, in order to develop a novel and effective anti-AML/LSC strategy.

Materials and Methods

Cells. HL60, KG1, THP-1 and NB4 leukaemia cell lines (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI-1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (HyClone Laboratories, Logan, UT, USA) and 1% penicillin/streptomycin. Primary leukaemia cells from patients with AML and bone marrow cells from healthy volunteers were obtained with informed consent in accordance with the Declaration of Helsinki. CD34-positive (CD34+) cells were isolated using MiniMACS system (Miltenyi Biotech, Auburn, CA, USA). The mean purity of the CD34+ fraction was 85%. All manipulations and analyses of specimens were approved by the Institutional Review Board of Yonsei University College of Medicine.

Key Words: Leukaemia stem cells, PI3K/Akt, CK2, synergism, targeted therapy, apoptosis.
Reagents and cell culture. Apigenin (Sigma, St. Louis, MO, USA) and LY294002 (Calbiochem, San Diego, CA, USA) were reconstituted in dimethyl sulfoxide (DMSO) to a stock concentration of 50 mM. The final concentration of DMSO in the cultures was kept at 0.1%. For all experiments, the cells (2×10^5/ml) were placed in each well of a 24-well plate containing 1 ml RPMI-1640 plus 10% FBS and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Control cells were treated with equal amounts of the solvent. All experiments were performed in triplicate.

Assessment of apoptosis. Annexin V/propidium iodide (PI) assay was done according to the manufacturer’s protocol (BD Biosciences, San Diego, CA, USA). The cells were washed with Dulbecco’s PBS (DPBS) without calcium or magnesium (Life Technologies), and incubated in 100 μl of a binding buffer containing 5 μl of annexin V-fluorescent isothiocyanate (FITC). The nuclei were counterstained with PI. The percentage of apoptotic cells was determined using FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with Cell Quest software and LY294002 (Calbiochem, San Diego, CA, USA). The cells were washed with Dulbecco’s PBS and centrifuged at 700 × g for 10 min at 4°C, the supernatant was collected and centrifuged at 10,000 × g for 30 min at 4°C to collect the cytosolic fraction. The mitochondrial fraction was obtained after dissolving the cell pellet in 100 μl mitochondria extraction buffer and vortexing for 10 s.

Transient transfection. For transfecting pcDNA3-Myr-Akt (a constitutively active Akt cDNA) and a specific CK2α siRNA to leukaemia cells, the Nucleofector system (Amx® Biosystems, Gaithersburg, MD, USA) was used, as previously described (15). Briefly, 5 μg of DNA or siRNA was mixed with 100 μl of appropriate Nucleofector solution, and a CI empty vector was used as a control in cDNA transfection experiments. CK2α siRNA (5’-GATGACACCA GCTGGTTC-3’) was obtained from Qiagen Inc. (Valencia, CA, USA). According to the manufacturer’s instructions, after electroporation by a Nucleofector instrument, 2×10^6 cells were immediately suspended in the complete medium and incubated in a humidified 37°C/5% CO_2 incubator.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA) and SPSS software version 11.0.1 (SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered to be statistically significant. Drug interactions for synergy was examined using median dose–effect analysis (Calcusyn; Biosoft, Ferguson, MO, USA). Unless otherwise indicated, all data were expressed as mean±standard deviation (SD), calculated from at least three independent experiments.

Results

Synergistic induction of apoptosis by LY294002 and apigenin in myeloid leukaemia cells. Apigenin or LY294002 by itself exerted an apoptotic effect on HL60 cells in a dose-dependent manner, but the apoptosis was minimal at or below 25 μM. However, the combination treatment of LY294002 and apigenin (LY/Api) at 25 μM remarkably induced apoptosis (Figure 1A and 1B). Robust cell death was evident early after 24 h of LY/Api at a subtoxic concentration of 25 μM (Figure 1C). Median dose–effect analysis was used to characterize interactions between LY294002 and apigenin with respect to the induction of apoptosis after 48 h of co-treatment and combination index values were consistently below 1. This result suggested a highly synergistic interaction between the two agents (Figure 1D).

This synergistic induction of apoptosis by LY/Api was documented in other leukaemia cell lines (Figure 2A). Mean
proportion of apoptosis of KG1, NB4 and THP-1 cells by LY/Api was 67.9±0.9%, 52.7±0.4%, and 72.5±0.8%, respectively, which was significantly higher compared to the respective apoptosis proportion induced by apigenin (p<0.01 for all cell lines) or LY294002 alone (p<0.01 for all cell lines) (Figure 2A). Both the mean proportion of apoptosis by LY/Api in primary leukaemia cells (72.2±1.8%) and in CD34+ blasts (92.3±1.6%) from patients with AML were also significantly higher compared to the respective apoptosis proportions induced by apigenin (10.9±1.0%, p<0.001; 21.5±4.9%, p<0.001, respectively), and LY294002 alone (14.6±1.2%, p<0.0001; 19.6±4.9%, p<0.001, respectively) (Figure 2B and 2C). However, in contrast to leukaemia cells, LY/Api did not induce apoptosis in healthy HSCs (Figure 2B lower panel and 2C).

**Preferential cytotoxicity of LY/Api on CD34+CD38− leukaemia cells.** To evaluate the effects of LY/Api against LSCs, the changes in the proportion and viability of CD34+CD38− AML cells caused by different treatments were examined. Apigenin and LY294002 at subtoxic concentration individually did not significantly affect the proportion and viability of CD34+CD38− leukaemia cells. However, LY/Api remarkably decreased the mean proportion of this population in the total AML cell population (Figure 3A, upper panel). The mean proportion of annexin V-7-AAD− viable CD34+CD38− leukaemia cells (20.6±2.5%) by LY/Api was also significantly lower than that by apigenin (81.8±4.2%, p<0.001), and LY294002 alone (74.7±4.9%, p<0.001) (Figure 3A lower panel and 3B). In contrast to CD34+CD38− leukaemia cells, LY/Api did not significantly change the mean proportion of annexin V-7-AAD− viable healthy HSCs (Figure 3C).

**LY/Api-induced apoptosis through the caspase-dependent mitochondrial pathway.** Next, the main mechanisms of LY/Api-induced apoptosis on HL60 cells were evaluated. The disruption of MMP after 24-h treatment was significantly increased in LY/Api-treated cells (47.2±1.8%), which was significantly higher than in apigenin-only-treated (2.8±0.2%, p<0.05) and LY294002-only-treated cells (1.9±0.1%, p<0.01).
This disruption of MMP was more prominent after 48 h, leading up to 80% of cells showing low Δψm. Western blot analysis was performed to monitor the expression of various proteins implicated in apoptosis. At 24 and 48 h of exposure intervals, LY/Api induced the cleavage of caspase-8, caspase-9, caspase-3 and PARP, as well as cytosolic release of cytochrome c and SMAC/DIABLO from mitochondria, but each inhibitor alone did not (Figure 4B and 4C). Treatment with caspase inhibitor z-VAD-fmk (20 μM) significantly abrogated apoptosis (63.0±8.7% vs. 14.5±1.2%, p<0.01) and the cleavage of caspase and PARP by LY/Api in HL60. These results indicated that LY/Api-induced apoptosis is dependent on the activation of caspase cascades (Figure 4D). The levels of anti-apoptotic molecules, such as Bel-xL, Mcl-1, XIAP and survivin, were also remarkably decreased in HL60 cells, primary AML cells and CD34+ AML cells by LY/Api, but not by each inhibitor alone (Figure 4E).

Effect of LY/Api on CK2α or Akt expression in leukaemia cells. After confirming that CK2α and Ser473 p-Akt expression in LSCs was significantly higher than in healthy HSCs (Figure 5A), the effect of LY/Api on CK2α or Akt expression in leukaemia cells was evaluated. In contrast to apigenin or LY294002 alone at subtoxic concentrations, LY/Api induced a remarkable decrease in the levels of constitutively activated CK2α and Ser473 p-Akt in both HL60 cells and primary AML cells (Figure 5B). Next, the effect of CK2α gene silencing on LY294002-induced apoptosis was examined. Similar to LY/Api, CK2α gene silencing induced a remarkable increase in the extent of LY294002-induced HL60 cell death (13.7±1.3% for control siRNA plus LY294002 vs. 44.3±3.0% for CK2α siRNA plus LY294002; p<0.05; Figure 5C). The effects of Akt overexpression on apoptosis were also analysed and the synergistic effect of LY/Api on inducing apoptosis was potentially abrogated by the induced overexpression of active Akt cDNA (Myr-Akt) in HL 60 cells (Figure 5D); the mean proportion of apoptosis in Myr-Akt-transfected cells (27.2±6.3%) was significantly lower than that in parental cells (65.3±3.3%, p<0.05) and in empty vector-transfected cells (72.6±4.0%, p<0.05). These findings together suggested that the synergistic induction of apoptosis by LY/Api occurs through selective inhibition of Akt and CK2α.

Effect of LY/Api on other pathways in leukaemia cells. To investigate what effects, if any, apigenin and/or LY294002 may have on other signal pathways in leukaemia cells, alterations of key molecules were evaluated. In contrast to each inhibitor alone, LY/Api for 48 h notably decreased the levels of Wnt1, β-catenin, LRP6, and Dvl in both HL60 and primary AML cells (Figure 6A). Similarly, apigenin and LY294002 individually did not affect the levels of p-Raf-1, p-MEK1/2 and p-ERK1/2, but LY/Api markedly decreased those levels in leukaemia cells (Figure 6B). Additionally, NF-κB levels in HL60, primary...
AML and primary CD34+ AML cells were also remarkably decreased by LY/Api, but not by apigenin or LY294002 alone (Figure 6C). In contrast, NF-κB levels were not altered, even with LY/Api combination, in healthy HSCs.

Discussion

The present findings indicated that the CK2 inhibitor, apigenin, interacts in a highly synergistic manner with the PI3K/Akt inhibitor, LY294002, to induce apoptosis in human myeloid leukaemia cells, especially in LSCs containing CD34⁺CD38⁻ cell populations, while sparing healthy HSCs. Activation of caspase cascades, disruption of MMP and down-regulation of molecules in the CK2α, PI3K/Akt and anti-apoptosis pathways accompanied the apoptosis induced by LY/Api.

Interaction between LY294002 and apigenin is noteworthy in several respects, including the molecular mechanism interrupting the multiple deregulated signal transduction pathways (STPs), the synergistic interaction at low concentrations of each agent, and most importantly, the

Figure 3. LY/Api-induced apoptosis observed preferentially in CD34⁺CD38⁻ leukaemia cells but not in normal HSCs. Primary AML, CD34⁺CD38⁻ leukaemia cells, and HSCs were exposed to each inhibitor alone or LY/Api for 48 h. A: The proportion of CD34⁺CD38⁻ leukaemia cells was markedly decreased only by LY/Api (upper panel). The scatter plot analysis (lower panel of A) and the analysis of mean proportion of apoptosis (B) demonstrated that the proportion of annexin V-positive apoptotic cells by LY/Api was significantly increased in CD34⁺CD38⁻ leukaemia cells (n=31) compared to that caused by apigenin alone (p<0.001) and LY294002 alone (p<0.001). C: In HSCs (n=7), LY/Api did not induce significant apoptosis compared to control and each inhibitor alone. Data represent mean±SD of three independent experiments.
preferential cytotoxicity on LSCs. In leukaemia cells, multiple STPs are frequently deregulated and constitutively activated by upstream mutations in cytokine receptors, aberrant chromosomal translocations and other genetic mechanisms (10, 11, 19-22). For this reason, a large number of molecular-targeted agents against AML have been introduced, but have showed limited or sporadic efficacy in clinical trials despite their potent in vitro effects (19, 23). One of the reasons for this limitation may be the simultaneous activation of multiple STPs via cross-activations or interconnections between each STP (10, 19, 24). Therefore, simultaneous inhibition of targets in more than two different STPs may help optimise the overall therapeutic benefit (21, 23). In fact, an emerging body of evidence suggests that leukaemic cells are highly susceptible to a therapeutic strategy disrupting multiple

Figure 4. Effects of LY/Api on caspase-dependent mitochondrial pathway and anti-apoptosis molecules. A: The disruption of mitochondrial membrane potential (ΔΨm) in HL60 cells was significantly increased after 24-48 h of LY/Api compared to apigenin (p<0.05) or LY294002 alone (p<0.05). Western blot analysis revealed that the cleavage of caspase-8, caspase-9, caspase-3 and PARP (B), as well as the translocation of cytochrome c and SMAC/DIABLO from mitochondria into the cytosol (C) was documented 24-48 h after LY/Api. D: Caspase inhibitor z-VAD-fmk (20 μM) significantly abrogated apoptosis (p<0.01) and the cleavage of caspase and PARP by LY/Api in HL60. E: Western blot analyses demonstrated that the levels of Bcl-xL, Mcl-1, XIAP, and survivin were markedly decreased after 48 h of LY/Api in HL60 cells, primary leukaemia cells and AML CD34+ cells. Data represent means±SD of three independent experiments. UPN: Unique patient number.
Figure 5. Effect of LY/Api on CK2α and Ser^{473} p-Akt expression in leukaemia cells. A: CK2α and Ser^{473} p-Akt expression was significantly higher in CD34^{+}CD38^{-} leukaemia cells compared to normal HSCs. B: In HL60 cells and primary AML cells, LY/Api induced a remarkable decrease in the levels of constitutively activated CK2α and Ser^{473} p-Akt. C: Western blot analysis showed that CK2α was effectively knockdown with CK2α siRNA transfection (upper panel), and CK2α gene silencing induced a remarkable increase in the extent of LY294002-induced apoptosis compared to control siRNA-transfected cells (lower panel, p<0.05). D: Induced Akt overexpression of transfection with active Akt cDNA (Myr-Akt) in HL 60 cells significantly abrogated LY/Api-induced apoptosis (p<0.05). Data represent mean±SD of three independent experiments. UPN: Unique patient number.
STPs simultaneously. Furthermore, tumour cells may be more sensitive than healthy cells to inhibition of STP because tumour cells are highly reliant on deregulated STPs for their survival (25-27). Thus, even a partial inhibition of these pathways may be sufficient to negatively affect neoplastic survival and proliferation while sparing healthy cells (25, 28, 29) and this effect may be enhanced when another inhibitor is added (30). Conceivably, a combination therapy would allow the use of lower dosage of both inhibitors, giving maximum efficacy on leukaemia cells and minimum side-effects on healthy HSCs. This finding is very similar to previous observations on the sensitisation of prostate cancer cells to induction of apoptosis by suboptimal doses of TRAIL in the presence of a small reduction in CK2 activity (25, 28).

The prototypes of the combined blockade include inhibition of parallel STPs (31-33), simultaneous targeting of STPs and apoptosis pathways (34-36) and intra-pathway inhibition of the upstream and downstream kinases (20, 37, 38). The last strategy is considered to be the most promising because there is growing proof that longitudinal inhibition of STPs is an effective strategy for combating reciprocal feedback loop activation between target molecules (37).

CK2 and PI3K/Akt were simultaneously inhibited in AML cells for several reasons. First, both PI3K/Akt and CK2α are frequently observed in primary AML cells and significantly associated with adverse prognosis in AML (10, 15). Secondly, PI3K/Akt sits at the crossroads of multiple oncogenes and tumour suppressor signalling networks (18, 39). Thirdly, a possible link has been suggested between these two protein kinases. The physical association of Akt with CK2 and the observation that CK2α enhances the kinase activity of Akt via direct phosphorylation at Ser129 (16) or via indirectly controlling PTEN activity (40) suggest the possibility that CK2 may act in concert with Akt to promote proliferation and protect cells from apoptosis. These findings provided the rationale for trying the simultaneous inhibition of both kinases to potentially induce cell death in myeloid leukaemia cells and more important, in apoptosis-resistant LSCs.

The underlying molecular mechanism for inducing apoptosis selectively in leukaemia cells by LY/Api is not yet clear. It should be emphasised that, given its extreme pleiotropy of CK2 and PI3K/Akt, LY/Api may affect numerous STPs (34, 41), eventually counteracting anti-apoptotic mechanisms through complex inhibition of
multiple targets. In this study, LY/Api was associated with a notable decrease in the levels of proliferation-associated and anti-apoptotic molecules such as p85-PI3K, Ser473-p-Akt/PKB, Bcl-xL, Mcl-1, XIAP, survivin and NF-κB. Considering the constitutive activation of NF-κB in LSCs but not in healthy HSCs, inhibition of NF-κB with LY/Api may also provide unique opportunities to ablate LSCs preferentially (42). In addition, LY/Api led to greater down-regulation of the Raf/MEK/ERK pathway, which may, in part, contribute to the synergistic antiproliferative effects, because the expression of both activated Raf/MEK/ERK and PI3K/Akt pathways may synergise and result in the abrogation of the cytokine dependence of the haematopoietic cells (43). Compared to other antileukaemia agents, the plurality of action of LY/Api potentially confers efficacy in a wide spectrum of AML, particularly in LSCs, which have heterogeneity and multiple defects in STPs. A recent study suggested that LSCs, through their HSCs-like properties, are responsible for disease progression or relapse (44) through drug resistance mechanisms, including expression of ATP-associated transporters (6, 7). It was shown that as many as 96% of the LSCs, defined by the phenotype CD34+CD38–CD123+, are in the G0 phase of the cell cycle (3). This resting status of LSCs protects them from the commonly used cell cycle-specific chemotherapeutics (6-8). In addition, conventional leukaemia therapy is substantially toxic to healthy HSCs and causes impairment of the haematopoietic function (9). Therefore, the present finding that LY/Api synergistically induced apoptosis of LSCs while sparing healthy HSCs may be a potential regimen for targeted therapy for LSCs in AML.

In summary, the results of the present study call for attention to a strikingly synergistic potentiation of cytotoxicity achieved by combining a CK2 inhibitor and a PI3K/Akt/PKB inhibitor in a panel number of human myeloid leukaemia cells and, more importantly, in CD34+CD38– leukaemia cells containing LSCs. It should also be noted that the effectiveness of this combination is accompanied by remarkable down-regulation of multiple targets implicated in leukaemia cell growth, anti-apoptosis and drug resistance. Since this finding was not observed in healthy HSCs, LY/Api may be a promising LSC-targeted therapeutic treatment in AML.

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References


