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LY294002 inhibits leukemia cell invasion and migration through early growth response gene 1 induction independent of phosphatidylinositol 3-kinase-Akt pathway

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ABSTRACT

LY294002 (LY29) is a commonly used pharmacologic inhibitor of the phosphatidylinositol 3-kinase (PI3 K) and has shown antitumorigenic effect both *in vivo* and *in vitro*. Both LY29 and its inactive analogue, LY303511 (LY30), significantly up-regulated early growth response gene 1 (Egr-1) expression in HL-60 leukemic cells. However, wortmannin, another commonly used PI3K inhibitor, was not able to induce Egr-1 at the dose that completely blocked Akt phosphorylation. LY29 markedly decreased the invasive cells number through Matrigel and human umbilical vein endothelial cells (HUVECs) compared with the controls. Moreover, the inhibitory effects could be significantly abolished by Egr-1 gene silencing with siRNA technology. Our results indicated for the first time that LY29 could suppress leukemia cell invasion and migration at least in part through up-regulation of Egr-1, independent of its PI3 K-Akt inhibitory activity. These data provide a novel explanation for the anticancer properties of LY29 in leukemias.

The phosphatidylinositol 3-kinase (PI3K)-Akt/PKB signaling is a well-characterized pathway involved in the control of cell proliferation, apoptosis, angiogenesis and oncogenesis [1,2]. Deregulated activation of this pathway, achieved through numerous genetic and epigenetic alterations, has been found in many types of cancer including leukemias [3–5]. LY294002 (LY29) is a commonly used pharmacologic inhibitor of PI3K, where it acts on the ATP-binding site of the PI3K enzyme, thus selectively inhibiting the PI3K-Akt nexus. LY29 has been shown to exert antitumorigenic effect *in vivo* and *in vitro* by induction of apoptosis and cell growth arrest [6,7]. Moreover, tumor cell invasion and migration could be significantly attenuated by LY29 treatment in several tumor models [8–10]. Until now, most studies on LY29 attributed its anticancer activity to the inhibition of PI3K pathway.

The early growth response gene 1 (Egr-1) is a member of the immediate-early gene group of transcription factors which modulate transcription of multiple genes involved in cell proliferation, differentiation, and transformation [11]. The overall cellular responses to Egr-1 are complex and dependent on both promoter- and cell-context. Several investigations have shown that Egr-1 play an important role in the control of tumor metastasis through regulation of cancer invasion related genes, including TGF-ß1, thrombospondin-1 (TSP-1), and plasminogen activator inhibitor-1 (PAI-1) [12,13].

Here, we report for the first time that LY29 reduces leukemia cell invasion and migration by a novel mechanism involving up-regulation of Egr-1 independent of its PI3K inhibitory activity.

Materials and methods

Cell culture and reagents. The human acute myeloid leukemia cell line HL-60 (American Type Culture Collection, Rockville, MD) was routinely grown in suspension in RPMI 1640 medium (Gibco, Grand Island, NY) containing glutamine (0.200 g/l), antibiotics (penicillin 100 IU/ml; streptomycin 100 μg/ml) and supplemented with 10% heat-inactivated fetal bovine serum (FBS), in a 5% CO_2 humidified atmosphere at 37 °C. Human umbilical vein endothelial cells (HUVECs) were cultured in Medium 199 Modified Earle's salts (Gibco), containing 15% FBS and 50 μg/ml endothelial cell growth factor. HUVECs were used up to the fourth passage. Cell viability was determined by using a trypan blue dye exclusion assay. LY29, LY303511 (LY30), and wortmannin were purchased from Sigma (St. Louis, MO).

Western blot. Proteins in cell lysates were resolved by SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in bovine serum albumin (3%) in Tris-buffered saline (TBS)–Tween 20 (0.05%) and incubated with antibodies against human Egr-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or phospho-Akt (Ser473) (Cell Signaling Technology, Beverly, MA), at 1:1000 dilutions followed by horseradish peroxidase-conjugated secondary antibody at 1:5000 dilutions. Detection was performed by using enhanced chemiluminescence (ECL).

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MTT assay. Cells for testing were incubated for 2 h with MTT by adding 20 μl of 5 mg/ml MTT into each well in 96-well plate. Cells were then washed with phosphate-buffered saline and solubilized with 100 μl dimethyl sulfoxide. Absorbance was read at $\lambda = 570$ nm and survival was calculated as a percentage of vehicle-treated controls.

Cell invasion and transendothelial migration assays. *In vitro* cell invasion and transendothelial migration assays were performed as described [14] with some modifications. For invasion assays, 5×10^5 HL-60 cells in RPMI/0.1%FBS were preincubated or not (control) for 3 h with LY29, and added to the upper chamber of 5 μm pore Transwell filters (Costar, Cambridge, MA) coated with Matrigel. Medium containing 20% FBS was added to the lower chamber as a chemoattractant. After 24 h at 37 °C, invasive cells were counted in flow cytometer. For transendothelial migration, 3×10^5 HUVECs were plated on fibronectin-coated Transwell filters and confluent monolayers were stimulated with 15 ng/ml TNF-α (Promega, Madison, Wis) for 16 h prior to the assay. HL-60 cells were added to the HUVECs monolayer and allowed to migrate towards the lower chamber filled with medium containing 20% FBS. After 24 h transmigrated cells were counted by flow cytometry. Cells that migrated through Matrigel or HUVECs were normalized to the total number of cells added, also counted by flow cytometry.

Quantitative real time RT-PCR. Total RNA was extracted from cells using Trizol (Invitrogen) and treated with DNaseI (Ambion). One microgram of total RNA was used for first-strand DNA synthesis using iScript cDNA Synthesis system (BioRad). Real-Time PCR was performed using iQ SYBR Green Supermix and the iCycler Real-Time PCR Detection System (BioRad) as described [15]. The following Primers were used for Egr-1 mRNA amplification: forward 5′-AGCCCTACGAGCACCTGAC-3′ and reverse 5′-TGGGTTGG TCATGCTCACTA-3′.

siRNAs. The siRNA sequences targeting Egr-1: sense 5′-AGAGG CAUACCAAGAUCCATT-3′, and the control siRNA sequence: sense 5′-CCUAGGUGCAUCCCGUUUG-3′, were custom-made by Ambion (Austin, TX). siRNA cell transfection was performed using the HiPerfect Transfection Reagent (Qiagen, Hilden, Germany) according to the recommended procedure. Transfected cells were used after 24 h.

Statistical analysis. Data are presented as means ± SD of at least three independent experiments. Differences between groups were analyzed using two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results and discussion

We first confirmed the PI3K-Akt inhibitory activity of LY29 at concentrations used in the current study. HL-60 leukemic cells were treated with increasing concentrations of LY29 (2–50 μM) for 3 h following 12 h preincubation in serum-free medium, and the effect on Akt/PKB activation was assessed by Western blot analysis. As shown in Fig. 1A, top panel, LY29 treatment effectively inhibited Akt phosphorylation at concentrations more than 5 μM. To determine whether LY29 could induce Egr-1 expression in leukemia cells, same samples were then detected for Egr-1 protein expression. As shown in Fig. 1A, middle panel, Egr-1 protein level was significantly up-regulated in HL-60 cells after 3 h LY29 incubation. The most robust Egr-1 protein induction appeared at 20 μM LY29 treatment and higher concentrations of LY29 resulted in

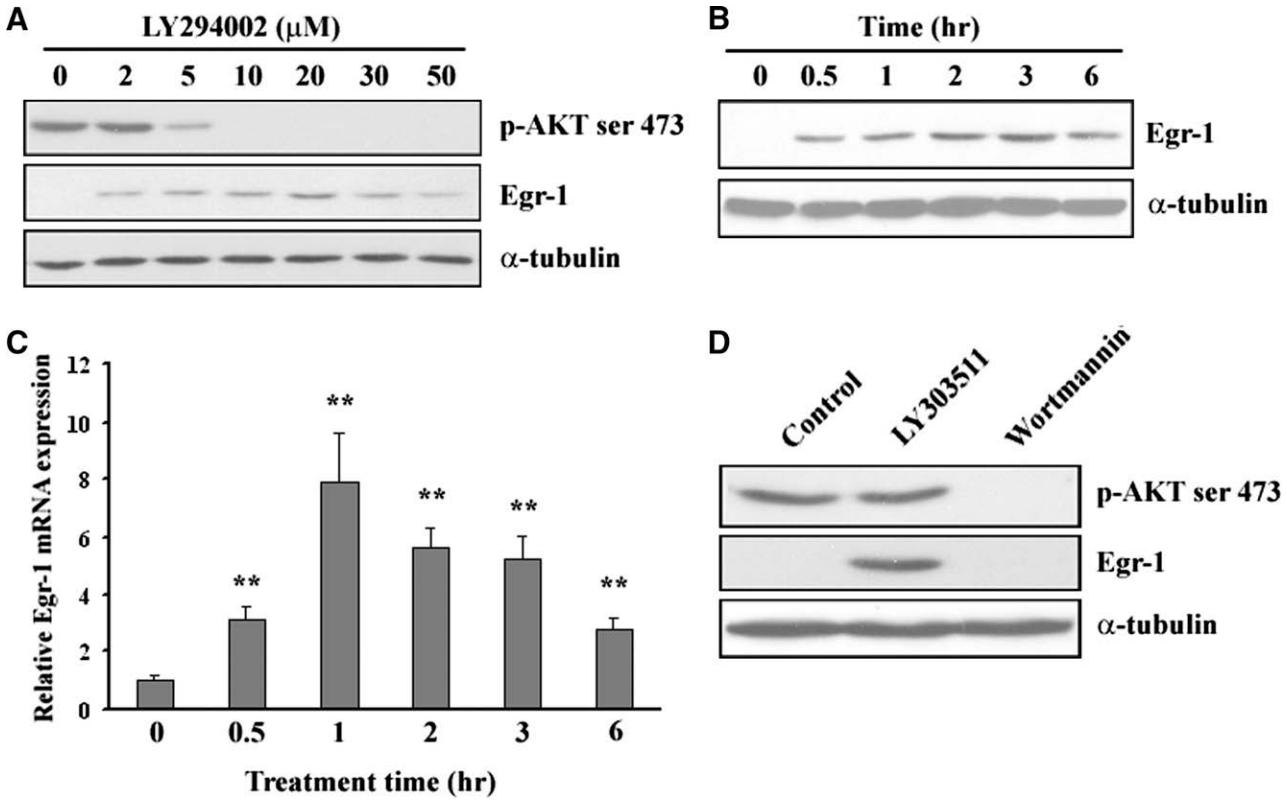

Fig. 1. Induction of Egr-1 expression by LY29 in a PI3K-Akt independent manner. A and B, HL-60 cells were incubated for 12 h in serum-free medium and treated with each dose of LY29 for 3 h (A) or 20 μM LY29 for each indicated time (B). Western blot was then performed to detect Egr-1 protein levels (A and B) and Akt phosphorylation status (A). The blots were stripped and re-probed with anti-α-tubulin antibody to show equal loading of total protein. (C) Total RNA was isolated from HL-60 cells after the same treatment as 1B, Egr-1 mRNA expression relative to 18S rRNA was determined by quantitative real time RT-PCR. (D) HL-60 cells were treated with 0.5 μM wortmannin or 20 μM LY30 for 3 h, Western blot was then performed to detect Egr-1 protein levels and Akt phosphorylation status. Results are shown as means ± SD of triplicates samples. $^{*}P < 0.05$, $^{**}P < 0.01$.

gradually decreased Egr-1 induction in HL-60 cells. Therefore, we selected this dose of LY29 in subsequent studies. HL-60 cells were also treated with 20 μM LY29 for the each indicated time and protein & mRNA levels of Egr-1 were measured by Western blot and real-time RT-PCR, respectively. As shown in Fig. 1B, LY29 treatment markedly increased Egr-1 protein level in HL-60 leukemic cells, with the highest induction following 3 h drug incubation. Maximal Egr-1 mRNA induction was detected at a time point as early as 1 h after exposure to LY29 (Fig. 1C).

One interesting phenomenon found in Fig. 1A is that LY29 was able to induce Egr-1 expression at a concentration (2 μM; Fig. 1A, middle panel) which could not affect Akt/PKB phosphorylation status, suggesting that its PI3K-Akt inhibitory activity is not necessary for Egr-1 induction. To address this question, wortmannin, another commonly used PI3K inhibitor, and LY30, an analogue of LY29 without any PI3K-Akt inhibitory activity were introduce to this study. As shown in Fig. 1D, wortmannin was not able to up-regulate Egr-1 expression at the dose that completely blocked Akt phosphorylation (0.5 μM). However, 20 μM LY30 significantly induced Egr-1 protein in HL-60 cells even though it could not change the PI3K-Akt activity. These data indicate that the induction of Egr-1 protein in HL-60 cells by LY29 is independent of its PI3K-Akt inhibitory activity.

To show the functional relevance of LY29-induced Egr-1 expression, we performed *in vitro* invasion and migration assay. For Matrigel invasion assays, 20 μM LY29 treatment significantly decreased the invasive cells number through Matrigel compared with the control. Moreover, this inhibitory effect could be markedly abolished by Egr-1 gene silencing with siRNA technology, while the control siRNA had no effect (Fig. 2A). Similarly, as shown in Fig. 2B, 20 μM LY29 treatment could significantly reduce the transendothelial migration ability of HL-60 cells. Egr-1 siRNA transfection dramatically attenuated the suppression of cell migration. LY29 at this concentration is not cytotoxic to HL-60 cells, as determined by MTT assay (data not shown). Thus, this inhibitory effect of leukemic cell invasion through Matrigel and HUVECs is attributable to up-regulation of Egr-1 by LY29. Egr-1 gene knockdown with siRNA was confirmed by immunoblot as shown in Fig. 2C.

Results of several studies showed that Egr-1 is involved in regulating invasive behavior of cancer cells. Induction of Egr-1 is associated with anti-metastatic and anti-invasive ability of β-lapachone in human hepatocarcinoma cells [16]. Egr-1 also mediates the inhibitory effect of cyclooxygenase inhibitors on tumor cell invasion in a lung adenocarcinoma model [17]. Sustained Egr-1 expression may lead to preferential inhibition of tumor cell invasion, angiogenesis and tumor growth [18]. For leukemia cells, Egr-1 has been reported to abrogate the block in terminal myeloid differentiation imparted by oncogenic c-Myc or E2F-1, suppressing their leukemia-promoting function in nude mice [19–21].

Even though LY29 has shown impressive anticancer activity in a number of *in vitro* and *in vivo* studies based on its negatively regulatory effect on PI3K, recent reports have suggested that this compound might have effects other than inhibiting this pathway. LY29 sensitize tumor cells to drug-induced apoptosis via intracellular hydrogen peroxide production independent of the PI3K-Akt Pathway [7]. LY29 suppresses monocyte chemoattractant protein-1

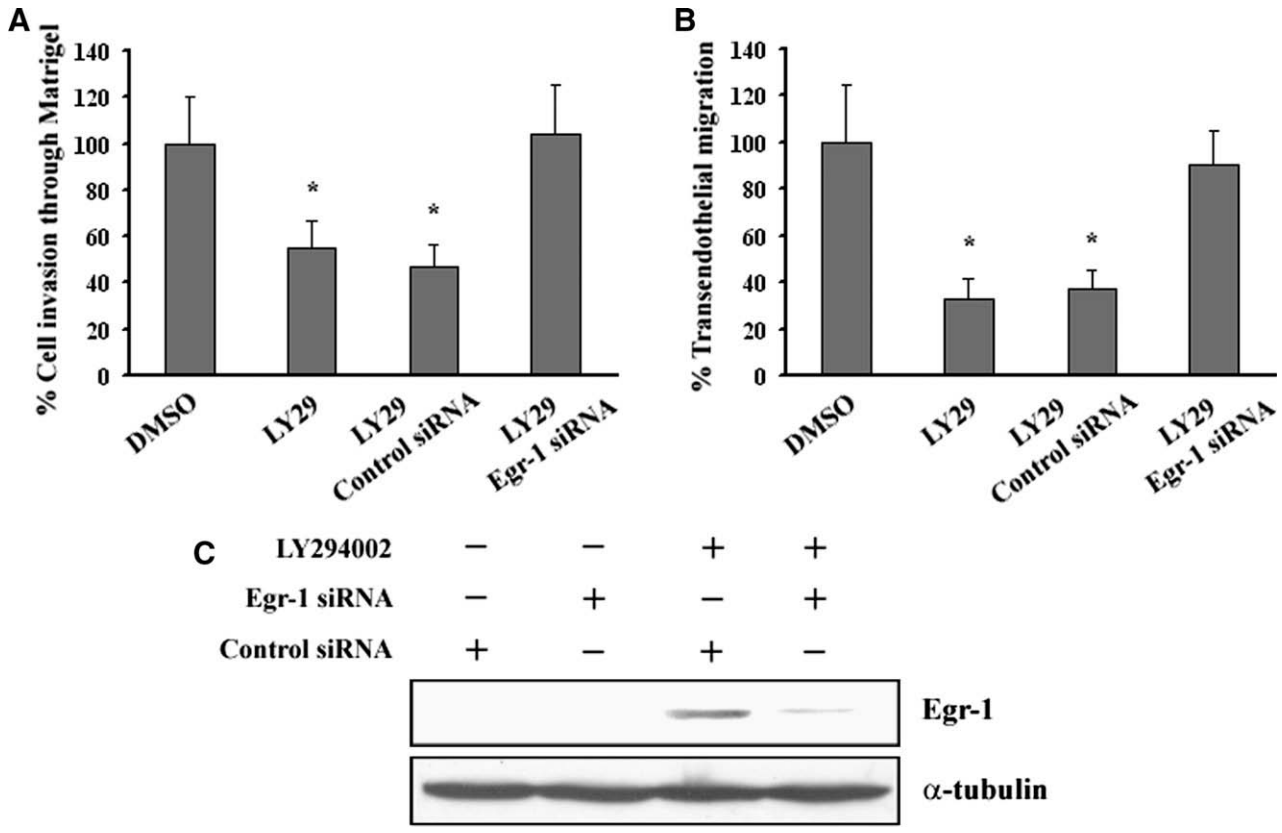

Fig. 2. Involvement of Egr-1 in inhibition of leukemic cell invasion by LY29. A and B, HL-60 cells (5×10^5) were pretreated with the indicated treatment (DMSO, 20 μM LY29 preincubation [3 h] only, LY29 preincubation plus control siRNA or Egr-1 siRNA transfection, respectively), then were added to the upper chamber of Transwell filters coated with Matrigel (A) or TNF-α-activated HUVECs (B). Medium containing 20% FBS was added to the lower chamber as a chemoattractant. After 24 h, invasive cells in the bottom chamber were counted by flow cytometry. Cells that migrated through Matrigel or HUVECs were normalized to the total number of cells added. Values represent means ± SD of triplicates samples and are expressed as fold differences relative to controls. (C) HL-60 cells were transfected with Egr-1 siRNA for 24 h followed by treatment with 20 μM LY29 for 3 h. Egr-1 protein level was analyzed by Western blot analysis. The blots were stripped and re-probed with anti-α-tubulin antibody to show equal loading of total protein. $^{*}P < 0.05$, $^{**}P < 0.01$.

expression by inhibiting the activation of nuclear factor-κB (NF-κB) without involving PI3K [22]. It sensitizes human leukemic cells to histone deacetylase inhibitor-mediated apoptosis through p44/42 MAP kinase inactivation and abrogation of p21 $^{CIP1/WAF1}$ induction rather than AKT inhibition [23].

The present study showed for the first time that LY29 could suppress leukemia cell invasion and migration at least in part through up-regulation of Egr-1, independent of its PI3K-Akt inhibitory activity. These data provide a novel explanation for the anticancer properties of LY29 in leukemias.

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