PI3K inhibitor LY294002, as opposed to wortmannin, enhances AKT phosphorylation in gemcitabine-resistant pancreatic cancer cells

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Abstract. LY294002 and wortmannin are chemical compounds that act as potent inhibitors of phosphoinositide 3-kinases (PI3Ks). Both of them are generally used to inhibit cell proliferation as cancer treatment by inhibiting the PI3K/ protein kinase B (AKT) signaling pathway. In this study, LY294002 (but not wortmannin) showed an abnormal ability to enhance AKT phosphorylation (at Ser472) specifically in gemcitabine (GEM)-resistant pancreatic cancer (PC) cell lines PK59 and KLM1-R. LY294002 was shown to activate AKT and accumulate phospho-AKT at the intracellular membrane in PK59, which was abolished by treatment with AKTi-1/2 or wortmannin. Inhibiting AKT phosphorylation by treatment with AKTi-1/2 or wortmannin further enhanced LY294002-induced cell death in PK59 and KLM1-R cells. In addition, treatment with wortmannin alone failed to inhibit cell proliferation in both PK59 and KLM1-R cells. Thus, our results reveal that LY294002 displays the opposite effect on PI3K-dependent AKT phosphorylation, which maintains cell survival from the cytotoxicity introduced by LY294002 itself in GEM-resistant pancreatic cancer cells. We suggest that targeting the PI3K/AKT signaling pathway with inhibitors may be counterproductive for patients with PC who have acquired GEM-resistance.

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Introduction

Phosphoinositide 3-kinases (PI3Ks) are a family of enzymes which are involved in cellular functions, such as cell growth, proliferation, differentiation and intracellular trafficking, which in turn contribute to cancer (1). Class I_A PI3Ks, which consist of a regulatory subunit (p85) and a catalytic subunit (p110), are frequently mutated in human cancers and many of these mutations cause the kinases to become more active (2-5).

PI3Ks can be activated by growth factor stimulation, which results in the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce phosphatidylinositol 3,4,5-bisphosphate (PIP_3) (6). PIP_3 directly binds phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (AKT), causing the colocalization of activated PDK1 with AKT, which allows AKT phosphorylation on threonine 308 (T308) by PDK1 (7,8). Complete activation of AKT occurs by phosphorylation on serine 473 (S473) by mammalian target of rapamycin (mTOR)-containing protein complex 2 (mTORC2) (9). PI3K/AKT signaling is closely associated to cellular proliferation and survival through several mechanisms, such as inhibition of pro-apoptotic B-cell lymphoma 2 (Bcl-2) family members BAD and BAX, activation of transcription factor NF-κB, suppression of p53 by phosphorylating E3 ubiquitin-protein ligase (Mdm2) and promotion of protein synthesis by stimulating mTORC1 (6,10,11).

Because inhibition of PI3K signaling can diminish cell growth and promote cell death, targeting the PI3K signaling pathway by inhibitors is being evaluated in clinical trials for cancer therapeutics. Preclinical studies have shown that PI3K pathway inhibitors have remarkable curative effects in several types of cancer, such as human epidermal growth factor receptor 2 (HER2)-amplified breast cancers, cancers with PIK3CA mutations and phosphatase and tensin homolog (PTEN)-deficient cancers (12-14).

Several reports have shown that PI3K inhibitors could be used as adjuvant therapy for pancreatic cancer, as it improved the anticancer drug efficacy by inhibiting the PI3K pathway and extended the overall survival in mouse models (15-18). In

this study, however, the PI3K inhibitor LY294002 has been shown to enhance PI3K-dependent AKT phosphorylation in pancreatic cancer cells. Interestingly, this phosphorylation induced by LY294002 only occurs once the cells have gained GEM-resistance. Moreover, inhibition of PI3K by treatment with wortmannin did not induce any apparent cytotoxicity in GEM-resistant cells. Our data indicate that the uncertainty and risk is still high for treating PC by using PI3K inhibitors.

Materials and methods

Cell lines. All cell lines were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, 05918, Gaithersburg, MD, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, 26140-079) and 21 mM of L-glutamine, at 37˚C, in a humidified 5% CO_2-95% air mixture. GEM-resistant cells derived from KLM1 (KLM1-R) was established as previous descriptions (19,20). KLM1 cells were tretaed with 10 µg/ml of GEM for 2 weeks, after which, cells were washed and resuspended in fresh medium. This was followed by a 2-week culture period which served as a recovery procedure. This procedure was repeated for another 3 cycles.

Materials. LY294002 (9901) and wortmannin (9951S) were purchased from Cell Signaling Technology (Boston, MA, USA). AKTi-1/2 (ab142088) was purchased from Abcam Biochemicals (Cambridge, MA, USA). Epithelial growth factor (E9644) was purchased from Sigma (St. Louis, MO, USA). The antibodies specific for p-ERK[T204/202] (sc-7383), ERK (sc-94200), Hsp27 (sc-13132) and actin (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies specific for Src (Src Antibody Sampler kit, 9935), p-Hsp27[S78] (2405), p-Hsp27[S82] (2401), PTEN (9552S), AKT (9272) and p-AKT[S473] (4058) were purchased from Cell Signaling Technology.

Western blotting. Total protein was extracted from the cells using lysis buffer (1% NP-40, 1 mM sodium vanadate, 11 mM PMSF, 501 mM Tris, 101 mM NaF, 101 mM EDTA, 1651 mM NaCl, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) (21). Equal amounts of protein (20 µg) were resolved by 5-20% SDS-polyacrylamide gel and then transferred onto PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA). The membrane was incubated with a primary antibody at 4˚C overnight and a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The immunoblots were visualized with a chemiluminescent reagent (Immunostar, Wako, Osaka, Japan) and detected by using an Image Reader LAS-1000 Pro (Fujifilm Corp., Tokyo, Japan).

Immunofluorescence. Cells were cultured on coverslips in 12-well plates at a density of 1x10^{5} cells per well. Cells were fixed using fresh 3.7% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100. After washing with PBS they were incubated in blocking solution (1% goat serum or 1% donkey serum in PBS with 0.1% Tween-20) for 1 h at room temperature (22). Cells

were treated with a primary antibody in blocking solution overnight at 4˚C and a secondary antibody for 1 h at room temperature. Cell nuclei were counter-stained with 1.43 µM DAPI (4,6'-diamidino-2-phenylindole). Confocal images were obtained by using Laser Scan Confocal Microscope (LSM 510 META; Carl Zeiss, Mobicity, Australia).

Cell proliferation assay. Cells were cultured in 96-well plates. After treatment, 20 µl of the 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) dye (Promega, Madison, WI, USA) was added to each well of the plate and incubated for 2 h at 37˚C, in a humidified 5% CO_2-95% air mixture. Optical density (OD) was read directly at 492 nm using the iMark™ microplate absorbance reader (Bio-Rad, Hercules, CA, USA). Each experiment was repeated three times.

Results

The PI3K inhibitor LY294002 (but not wortmannin) enhances AKT phosphorylation in PK59 cells. The effect of the PI3K inhibitor, LY294002, on AKT phosphorylation at serine 473 in PC PK59 cells was detected by western blot analysis. Unexpectedly, p-AKT was significantly increased rather than decreased by treatment with LY294002 for 24 h in a dose-dependent manner and over a time course (Fig. 1A and C). LY294002 was also shown to enhance epithelial growth factor (EGF)-triggered AKT phosphorylation (Fig. 1B). However, the other PI3K inhibitor, wortmannin, showed the expected performance in inhibiting the PI3K/AKT pathway (Fig. 1B and C). Interestingly, the effect of wortmannin on inhibiting AKT phosphorylation was weakened after 6 h of treatment and completely disappeared after 24 h (Fig. 1C). In addition, the phosphorylation of tyrosine-protein kinase CSK (Src), extracellular regulated protein kinases (ERK) and heat shock protein 27 (Hsp27), which have been shown to be involved in AKT activation (23-26), were not altered by treatment with LY294002 (Fig. 1D). These results indicate that LY294002, but not wortmannin, displays the opposite function to that expected in regulating the PI3K/AKT signaling pathway in PK59 cells.

LY294002-induced AKT phosphorylation specifically occurs in GEM-resistant PC cells. LY294002, as well as wortmannin, have shown their ability to inhibit PI3K/AKT activity in HeLa and HEK293 cells (Fig. 2A and B). LY294002 could also inhibit AKT phosphorylation in various types of PC cancer cell lines, such as in Panc-1, MIA-PaCa-2, AsPC-1 and BxPC-3 cells (27-29). We previously reported that PK59 is much less sensitive to GEM (IC_{50}, 294.72 µg/ml) than Panc-1 (IC_{50}, 8.07 µg/ml), MIA-PaCa-2 (IC_{50}, 6.81 µg/ml), AsPC-1 (IC_{50}, 1.05 µg/ml) or BxPC-3 (IC_{50}, 6.67 µg/ml) cells (30). We thus tested whether LY294002-induced AKT phosphorylation specifically occurs in GEM-resistant PC cells. A GEM-resistant PC cell line KLM1-R has been established by culturing the gemcitabine-sensitive KLM1 cells with 10 µg/ml of GEM (19,20), as shown (Fig. 2C). As hypothesized, upregulation of p-AKT instead of downregulation was observed by treatment with LY294002 in GEM-resistant KLM1-R compared to KLM1 cells in a dose-dependent manner (Fig. 2D). These
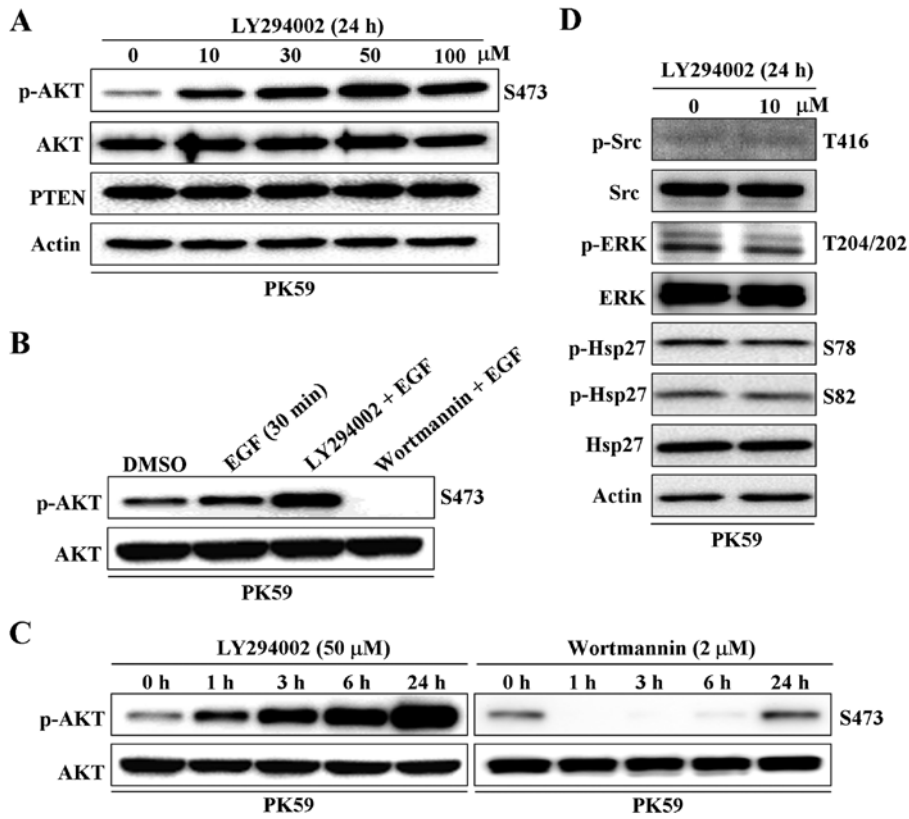
Figure 1. LY294002 induces upregulation of p-AKT in PC PK59 cells. (A) PK59 cells were treated with different dose of LY294002 for 24 h. (B) PK59 cells were treated with 1 μg/ml of EGF for 30 min in the presence of 50 μM of LY294002 or 2 μM of wortmannin. (C) PK59 cells were treated with LY294002 or wortmannin in a time course. (D) PK59 cells were treated with 10 μM of LY294002 for 24 h.
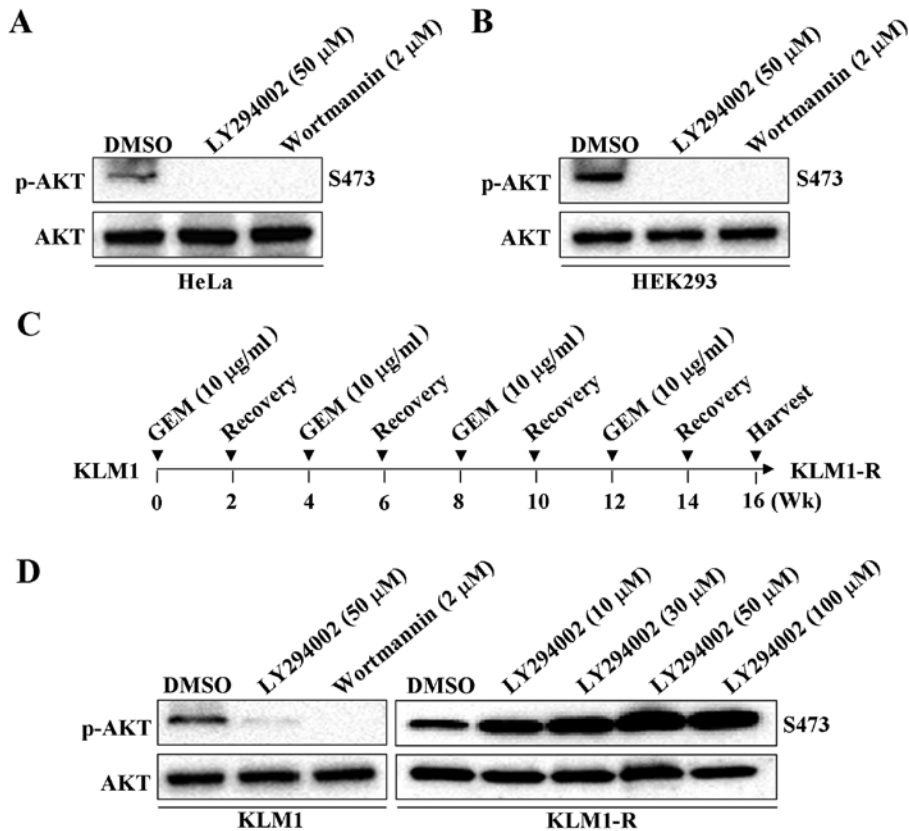

Figure 2. LY294002 induces AKT phosphorylation specifically in GEM-resistant PC cells. (A and B) HeLa and HEK293 cells were treated with 50 μM of LY294002 or 2 μM of wortmannin for 6 h. (C) The scheme indicates a process of the acquirement of GEM-resistance for PC KLM1 cells. (D) KLM1 and KLM1-R cells were treated with LY294002 or wortmannin for 6 h.
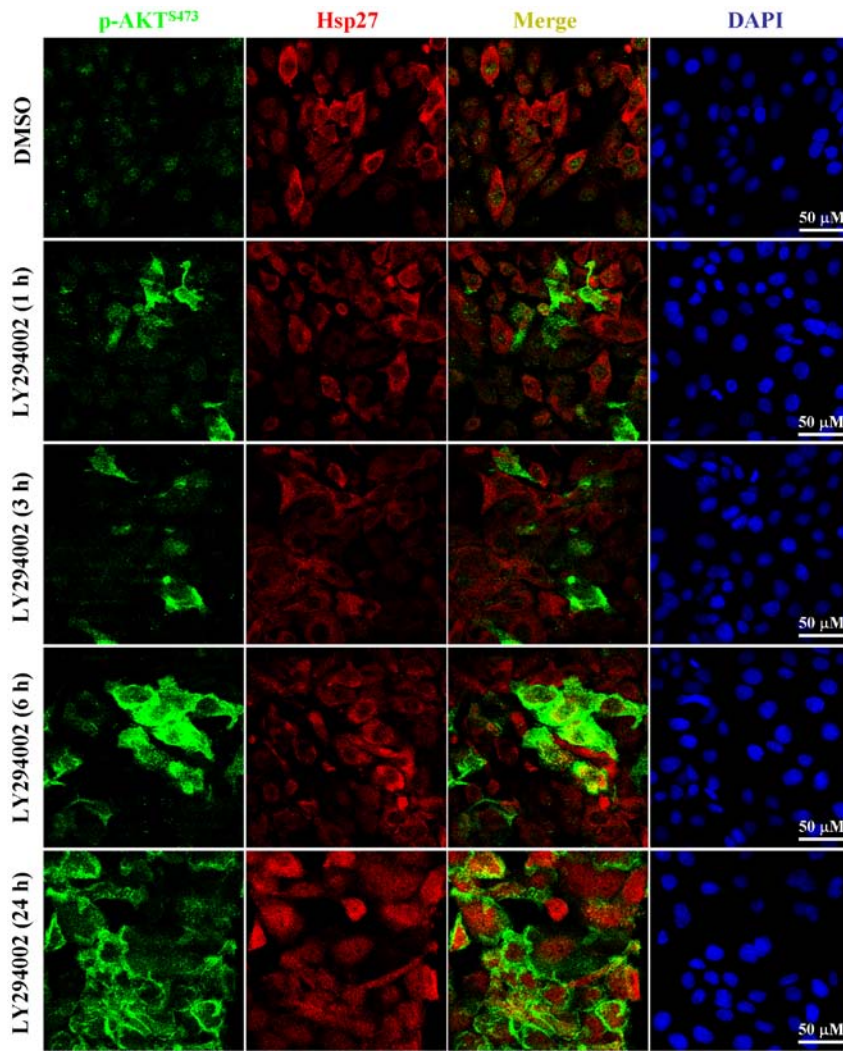
Figure 3. LY294002 induces the membrane localization of p-AKT. Immunofluorescent analysis was performed with anti-p-AKT and Hsp27 antibody by confocal microscope after cells were treated with 50 μM of LY294002 in a time course. Green, p-AKT; red, Hsp27; blue, DAPI.
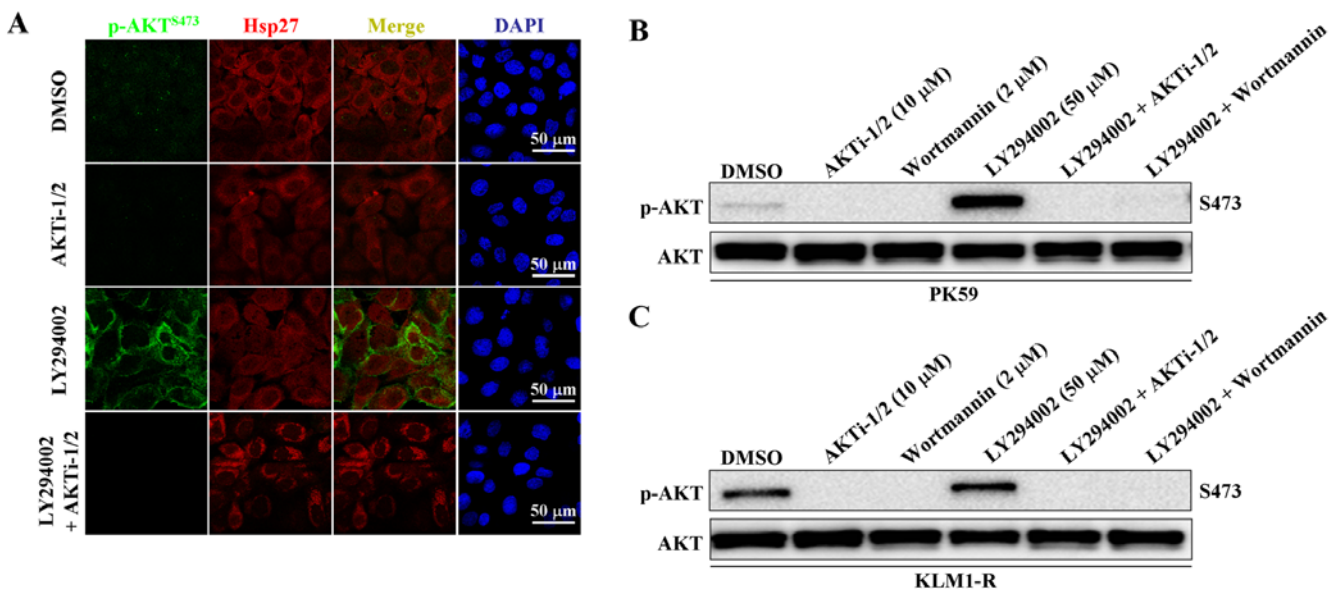

Figure 4. LY294002-induced AKT phosphorylation depends on PI3K. (A) Immunofluorescent analysis was performed with anti-p-AKT and Hsp27 antibody by confocal microscope after cells were treated with either 50 μM of LY294002 or 10 μM AKTi-1/2, or both for 24 h. (B and C) PK59 and KLM1-R cells were treated with LY294002 alone or combined with either wortmannin or AKTi-1/2 for 3 h.
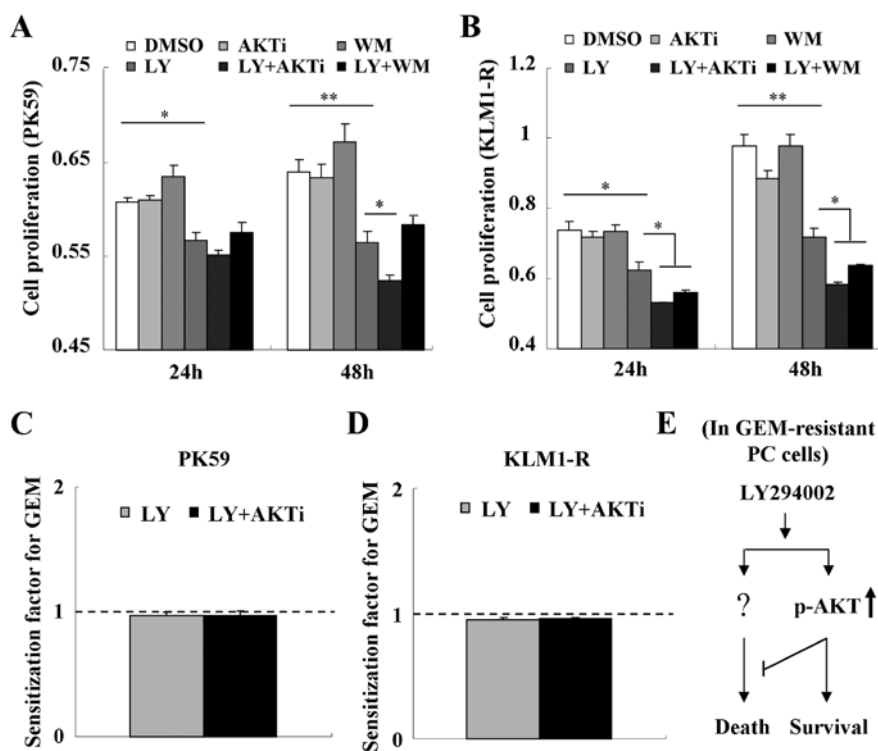
Figure 5. LY294002-induced AKT phosphorylation is associated with cell survival. (A and B) Proliferation assays were performed after PK59 and KLM1-R cells were treated with LY294002 alone or combined with either wortmannin or AKTi-1/2. **p<0.01; ***p<0.001. (C and D) Proliferation assays were performed after PK59 and KLM1-R cells were treated with LY294002 alone or combined with AKTi-1/2 in the presence or absence of 10 μg/ml of GEM. Sensitization factor indicates the ratio of cell viability (GEM/GEM + LY) or (GEM/GEM + LY + AKTi). (E) The scheme indicates the pathway of LY294002 on p-AKT in GEM-resistant PC cells. WM, wortmannin; LY, LY294002; AKTi, AKTi-1/2.

results suggest that the inhibiting ability of LY294002 on PI3K could be altered in PC, once cells acquire GEM-resistance. PI3K activity is required for the LY294002-induced AKT phosphorylation and associated intracellular membrane translocation.

Using immunofluorescent analysis, p-AKT could only be observed in the nuclei of untreated PK59 cells (Fig. 3). However, AKT phosphorylation was initiated in the cytoplasm of LY294002-treated cells and the p-AKT eventually translocated onto the intracellular membrane after treatment for 24 h (Fig. 3), indicating that the LY294002-induced p-AKT may have functions associated with this specific localization. This kind of phosphorylation and intracellular membrane translocation of AKT could be abolished by combined treatment with an AKT specific inhibitor, AKTi-1/2 (Fig. 4A). We next examined whether LY294002-induced AKT phosphorylation is mediated by PI3K. Following treatment with LY294002 alone, p-AKT was significantly upregulated, but following combined treatment with LY294002 and either AKTi-1/2 or wortmannin, p-AKT was completely inhibited in both PK59 and KLM1-R cells (Fig. 4B and C). These data reveal that LY294002-induced AKT phosphorylation and intracellular membrane translocation are still dependent on PI3K.

LY294002-induced AKT phosphorylation contributes to cell survival. We next assessed the role of LY294002-induced p-AKT in cell proliferation. Treatment with wortmannin was shown to increase or have no effect on cell proliferation in PK59 and KLM1-R cells, respectively (Fig. 5A and B), indicating that

GEM-resistant PC cells may also be resistant to the inhibitor of PI3K. Treatment with LY294002 alone significantly inhibited cell proliferation in PK59 and KLM1-R cells and this inhibition could be further enhanced by combined treatment with either AKTi-1/2 or wortmannin (Fig. 5A and B). These results indicate that LY294002-induced p-AKT plays a negative role in LY294002-triggered cytotoxicity in GEM-resistant PC cells. In addition, LY294002-induced p-AKT did not contribute to reducing the sensitivity of PK59 or KLM1-R cells to GEM (Fig. 5C and D).

Discussion

LY294002 is a synthetic compound that was designed as a PI3K inhibitor based on the flavonoid quercetin (31). Like most other protein kinase inhibitors, the mode of action of LY294002 is through competition with ATP for binding the PI3K active site (32). However, while quercetin is a broad-spectrum protein kinase inhibitor, LY294002 acts as a specific inhibitor for PI3K, with no inhibitory effect on other protein kinases such as the AMP-dependent protein kinase and c-Src (at a concentration of 50 mM) (31). Apart from PI3K, LY294002 has been shown to inhibit several key signaling components, such as NF-κB (33), heat shock proteins 27 (HSP27) and 72 (HSP72), AKT phosphorylation and survivin (34).

Interestingly, LY294002 binds PI3K in a different orientation to quercetin by a 180° rotation. When binding PI3K, LY294002 only occupies part of the active site of the catalytic domain, filling the space occupied by the ribose of the ATP

molecule, without extending into the phosphate binding region. In comparison, wortmannin almost completely fits the active site of the catalytic domain of PI3K, inducing a fairly large conformational rearrangement. Moreover, wortmannin forms a covalent complex, which irreversibly inhibits PI3K (35). The covalently-linked complex is the reason why in this study a decrease in wortmannin activity over time was observed, as the irriversibly bound complex was degraded and free PI3K was replenished.

LY294002 is a promising therapeutic compound because it showed growth inhibitory effects on various types of cancer cells through the induction of apoptosis and cell cycle arrest or autophagy (36,37). Apoptotic cell death by LY294002 is mainly driven via activation of AMPKα1 and inactivation of AKT (38), but can also be via NF-κB (33). However, it can also act by reducing transforming growth factor β (TGFβ)-mediated epithelial to mesenchymal transition (EMT) (39), inhibiting fatty acid synthase or suppress metastasis (40,41).

The available literature for the combinatorial treatment of pancreatic cancer by LY294002 and gemcitabine is plentiful. In pancreatic cancer cells, partial reversal of the EMT was accompanied by inhibition of cell invasion and migration as well as increased chemosensitivity to GEM, and the process was mediated by the phosphatidylinositol 3-kinase (PTEN)/ Akt signaling pathway (42). However, no importance appears to have been previously given to the effect of GEM-resistance to such treatment. The same can be said for most other flavonoids tested. One exception has been the treatment of GEM-resistant cell lines with GEM and apigenin (an isoconformer of the isoflavonoid genistein), which showed additive inhibition of cell proliferation compared to the use of either agent alone, via increased cell cycle arrest (at both the S and G2/M phases) as well as early apoptosis (by downregulation of pAkt) (43).

In conclusion, this is the first report to show that treatment of the GEM-resistant pancreatic cancer cell line PK59 with LY294002 (but not wortmannin) enhanced AKT phosphorylation specifically via PI3K and resulted in an associated translocation to the cell membrane. The overall effect of this LY294002-induced AKT phosphorylation on PK59 cells was an increase in cell survival. This should be an eye-opener for future clinical trials where the GEM-sensitivity of the tumor is not known.

Acknowledgements

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