

Inhibition of phosphatidylinositol 3-kinase sensitizes ovarian cancer cells to carboplatin and allows adjunct chemotherapy treatment

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Abstract

Signal transduction pathways associated with cancer progression and chemotherapeutic resistance are being investigated as molecular targets for chemotherapy. The phosphatidylinositol 3-kinase (PI3K) pathway has been found to be frequently amplified and have increased activity in ovarian cancer. The current study investigates the efficacy of an antagonist of PI3K, LY294002, in inhibiting ovarian cancer cell growth and survival both *in vitro* and *in vivo*. The hypothesis tested is that inhibition of PI3K signaling makes ovarian cancer cells susceptible to the effects of platinum-based chemotherapy. Observations show that LY294002 is an effective inhibitor of ovarian cancer cell growth and survival *in vitro*. Inhibition of PI3K/Akt signaling increased the sensitivity of ovarian cell cultures to the cytotoxic effects of carboplatin. The combined treatment of LY294002 and carboplatin was needed to optimally promote cellular apoptosis and decrease ovarian cancer cell survival *in vitro*. To extend these observations, a model involving *in vivo* i.p. growth of human ovarian tumors in a nude mouse was used. LY294002 in combination with carboplatin was more effective in inhibiting ovarian cancer cell xenograft growth than either agent alone. The results of this study suggest that the combined treatment of carboplatin and LY294002 can effectively decrease ovarian tumor progression and support the use of a PI3K inhibitor (e.g., LY294002) as an adjunct platinum-based drug therapy for treatment of ovarian cancer. [Mol Cancer Ther 2005;4(11):1764–71]

Introduction

The majority of ovarian cancers arise from the single layer of cells that surrounds the ovary, termed the surface epithelium (1–3). Epithelial ovarian cancer is the fourth leading cause of cancer death in women and the number one cause of death from gynecologic malignancy (1–3). As there are no overt symptoms associated with its onset and no current reliable methods of early detection, most ovarian cancers are not discovered until they have progressed to advanced stages (1–4). The current regimen of chemotherapy for ovarian cancer consists of a combination of either cisplatin or carboplatin and paclitaxel (4–6). Although these compounds have improved treatment success rates over the past decade, the majority of patients experience a relapse, and in most patients, the disease persists (4–6). The late-stage diagnosis and ineffective treatment contribute to a very poor prognosis for women with ovarian cancer. Therefore, the development of a more effective chemotherapy treatment would be instrumental in the ability to fight ovarian cancer.

An improved understanding of the molecular biology of ovarian cancer has led to the elucidation of potential therapeutic targets (7, 8). Previous studies have shown that the gene *PIK3CA*, which encodes the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), is increased in copy number in ~80% of primary ovarian cancer cells and in several ovarian epithelial carcinoma cell lines (9). PI3K is a heterodimeric kinase that is composed of a catalytic subunit (p110) and an adaptor/regulatory subunit (p85) and is activated by both receptor tyrosine kinases and G-protein-coupled receptors (10–12). Activated PI3K is able to phosphorylate the inositol ring 3'-OH group in inositol phospholipids and generate the second messenger phosphatidylinositol-3,4,5-triphosphate. The generated membrane phospholipids are responsible for recruitment of the serine/threonine kinase Akt to the plasma membrane and its subsequent phosphorylation and activation (10, 11). As a broad range of functions related to cancer progression are associated with PI3K activity, including proliferation, cell adhesion, apoptosis, and transformation (11, 12), it is not surprising that a gain-of-function mutation in this gene product would be involved in the etiology of ovarian cancer.

The downstream target, Akt, directs many of the actions attributed to PI3K activation and as such is a key regulator of cell survival and cell growth (13, 14). Interestingly, this downstream effector for PI3K is amplified or exhibits increased activity in a significant number of ovarian cancers (15). It has been shown recently in ovarian cancer cells that PI3K regulates G₁ cell cycle progression and cyclin expression through activation of Akt/mammalian

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target of rapamycin/p70S6K1 signaling pathway (16). Akt promotes cell survival through a variety of mechanisms, including phosphorylation and inactivation of proapoptotic proteins Bad and caspase-9 (13, 14). Phosphorylation of caspase-9 inhibits its protease activity and ability to activate caspase-3 (17).

A new approach for cancer treatment is the correction of specific genetic defects responsible for biological behavior of cancer cells (8, 18, 19). Signal transduction molecules that mediate cell fate decisions have come under scrutiny as potential targets for cancer therapy as their perturbation affords cancer cells increased growth potential and the ability to avert apoptosis (18, 20). In this regard, the PI3K/Akt signal transduction pathway is a good candidate for treatment in many cancer types (11, 21). It may be especially important for ovarian cancer because of its aberrant activity in this disease.

The flavonoid derivative, LY294002, is a competitive and reversible inhibitor of the ATP-binding site of PI3K (21, 22). LY294002 is effective in promoting apoptosis and blocking proliferation of different cancer cell types *in vitro* (21, 23, 24). Several studies have shown that inhibition of PI3K signaling by LY294002 induces apoptosis in ovarian cancer cells, exhibiting increased PI3K/Akt activation *in vitro* (9, 15, 25). Interestingly, studies by Hu et al. (26) show that LY294002 alone inhibits cell growth and induces apoptosis of ovarian cancer cells in culture and significantly decreases ovarian tumor xenograft growth in athymic mice.

Drug resistance to currently used chemotherapeutics is thought to be partly mediated by the ability to circumvent apoptosis (20, 27, 28). If chemotherapeutic-induced DNA damage accumulates beyond a threshold, programmed cell death will be initiated (4, 27, 28). In this regard, aberrant signaling through the PI3K pathway is thought to contribute to resistance to cisplatin (or carboplatin) and paclitaxel (21, 28). A recent study showed that cisplatin treatment of ovarian cancer cells induced Akt activation and inhibition of the Akt signaling increased the ability of cisplatin to initiate cell death (25). A separate study showed that overexpression of PI3K in ovarian cancer cells decreases sensitivity to paclitaxel and further shows that inhibition of PI3K activity by LY294002 sensitized ovarian cancer cells to the cytotoxic effects of paclitaxel both *in vitro* and *in vivo* (29). Alterations in ovarian cancer cell apoptosis by the PI3K/Akt pathway is a factor in chemotherapy resistance (30–32). In addition to effects on cellular apoptosis, effects of altered PI3K on ovarian tumor angiogenesis and vascular endothelial growth factor actions is another cellular process involved in chemotherapy resistance (33, 34). Studies with other types of cancer cells also indicate that alterations in the PI3K/Akt signal transduction pathway can modulate sensitivity to chemotherapeutic agents, including the response of small cell lung cancer to etoposide and acute myeloid leukemia cells to the DNA synthesis inhibitor 1- β -D-arabinofuranosylcytosine (23, 24).

The current study was designed to test the hypothesis that inhibition of PI3K signaling renders ovarian cancer cells susceptible to the effects of platinum-based chemotherapy.

The human ovarian cancer cell line, OCC1, was chosen for these studies because these cells display platinum-based drug resistance and exhibit an increase in *PIK3CA* copy number (9, 35). Additionally, xenografts of these cells form i.p. tumors in immunocompromised mice. For both *in vitro* and *in vivo* experiments, OCC1 cells have been stably transfected with the reporter gene, human *secreted alkaline phosphatase* (*SEAP*; ref. 36). The *SEAP* marker gene protein is constitutively expressed by these tumor cells and secreted levels can be directly correlated with tumor cell number and body tumor burden. A previous study showed that *SEAP* reporter gene expression can be used in a mouse model to monitor tumor progression and response to chemotherapeutics (36). This model makes it possible to monitor tumor progression over time, which is in contrast to other nude mouse studies where animals must be sacrificed after treatment to assess tumor burden (26). In the present study, we show that the combination of the PI3K inhibitor LY294002 and carboplatin is more effective than either agent alone in inhibiting OCC1 ovarian cancer cell growth and survival both *in vitro* and *in vivo*.

Materials and Methods

Cell Culture

The human ovarian cancer cell line OCC1 was generously provided by Dr. Gordon Mills (M.D. Anderson Cancer Center, Houston, TX) and cultured under recommended conditions. The OCC1 cells were modified to constitutively express the marker gene, human placental *SEAP* (36). The *SEAP* gene encodes a heat-stable protein that is secreted in proportion to cell number (36). The OCC1 cells were stably transfected by Fugene reagent with a pCMV-*SEAP* plasmid. The clonal isolate that produced high levels of *SEAP* (OCC1-*SEAP*-12) was used in subsequent *in vitro* and *in vivo* experiments. The OCC1-*SEAP*-12 cells were grown in Ham's F-12 medium (Life Technologies, Grand Island, NY) plus 10% bovine calf serum (BCS). Once cells reached confluence, they were trypsinized and subcultured into appropriate plates.

Growth Assays

Cell proliferation was analyzed by determining the amount of [³H]thymidine incorporation into newly synthesized DNA. The OCC1-*SEAP*-12 cells were plated in 24-well plates in Ham's F-12 medium plus 10% BCS and allowed to reach 50% to 70% confluence. Following a 48-hour serum starvation, the culture medium was changed to DMEM plus 0.1% bovine serum albumin (BSA) and 0.1% BCS containing either vehicle control or carboplatin (0–100 mg/mL) alone or in combination with LY294002 (0–20 mmol/L). Treatments were removed after 18 hours and cells were incubated for 4 hours in medium containing 5 mCi/mL [³H]thymidine. Medium was removed and cells were disrupted by sonication in PBS. An aliquot of the sonicated solution of PBS was loaded onto a DEAE filtration plate (Millipore, Bedford, MA) and individual filters with bound DNA were collected for scintillation counting. Data were normalized to total DNA per well and determined by a SYBR Green fluorescent assay (36).

Cell Survival

Cell survival was assessed as cell number remaining in culture following exposure to treatments. The DNA content of individual culture wells was used as an indication of cell number. Cells plated in 24-well culture plates were allowed to approach confluence (80%) in Ham's F-12 medium plus 10% BCS. Cultures were then incubated in DMEM plus 0.1% BSA and 0.1% BCS in the presence of vehicle control, carboplatin (0–100 mg/mL), LY294002 (0–20 mmol/L), or a combination of these for 24 to 96 hours. Media aliquots were taken when appropriate for SEAP analysis. The DNA was measured fluorometrically as described previously (36). Briefly, the fluorescence of aliquots of sonicated cell suspensions into which SYBR Green I fluorescent dye (Molecular Probes, Eugene, OR) had been incorporated was measured.

DNA Isolation and Analysis

Following 48- to 72-hour treatment incubations, cells were suspended into culture medium and pelleted in tubes. DNA was isolated from collected cells using a Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN). The quantity and purity of nucleic acid preparations were estimated by measuring the absorbance of each sample ($A_{260\text{ nm}}/A_{280\text{ nm}}$). DNA preparations (10 mg/well) were loaded onto 1.2% agarose gels and visualized with ethidium bromide stain.

Western Blot Analysis

The OCC1-SEAP-12 cells were grown to 80% confluence. Cultures were incubated in DMEM plus 0.1% BSA and 0.1% BCS containing either vehicle control, carboplatin (0–50 mg/mL), LY294002 (0–20 mmol/L), or combinations of these treatments. Following 24, 48, or 72 hours, cells were lysed with sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, 0.003% glycerol, 0.5% β -mercaptoethanol]. Total cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were immunoblotted with antibodies to both phosphorylated and nonphosphorylated forms of Akt (Cell Signaling Technology, Beverly, MA) or with antibodies to cleaved and full-length caspase-3 (Cell Signaling Technology).

Nude Mouse Tumor Model

Athymic nude mice (NCR *nu/nu*) were either purchased from Taconic (Germantown, NY) or bred in-house at Washington State University. The OCC1-SEAP-12 cells were collected in HBSS and counted before injection. Treatments were initiated 7 to 10 days following an i.p. inoculation of mice with 1×10^7 OCC1-SEAP-12 cells. Stock solutions of carboplatin (paraplatin; Bristol-Myers Squibb, Princeton, NJ) and LY294002 were prepared in sterile filtered PBS and DMSO, respectively. Animals received i.p. injections of either vehicle control (PBS containing 25% DMSO) or carboplatin (60 mg/kg) alone or in combination with LY294002 (50 mg/kg) every other day for 6 days. Blood samples were collected into capillary tubes from saphenous vein lancements at regular intervals during and following treatments. Capillary

tubes were centrifuged and the plasma samples were frozen (-20°C) until the time of SEAP assay. The Washington State University Animal Care and Use Committee approved all procedures.

SEAP Assay

Blood plasma and cell culture medium samples were assayed for SEAP activity using the Great EscAPe SEAP Fluorescence Detection kit (Clontech Laboratories, Palo Alto, CA) as described previously (36). Blood plasma samples were diluted 1:100 before the assay to bring values within the linear range of the standard curve. The intraassay and interassay coefficients of variation were 2.5% and 18.8%, respectively.

Statistical Analysis

Data were analyzed by one-way ANOVA. Significant differences between treatment groups were determined using a Student's *t* test. Data were expressed as mean \pm SE.

Results

Ovarian cancer cells characteristically grow independent of growth factor stimulation. Basal levels of DNA synthesis of OCC1-SEAP-12 cells were blocked by 50% in cultures incubated in the presence of LY294002 and by 51% and 59.5% following treatment with 20 and 50 mg/mL carboplatin, respectively (Fig. 1). The combined treatment of LY294002 and 20 mg/mL carboplatin did not reduce DNA synthesis more than either treatment alone (Fig. 1). The reduction in [^3H]thymidine incorporation was not significantly different than that seen following treatment with 50 mg/mL carboplatin alone or in combination with LY294002 (Fig. 1). Observations show that PI3K inhibition results in decreased OCC1-SEAP-12 [^3H]thymidine incorporation to the same degree as seen with the chemotherapeutic agent, carboplatin.

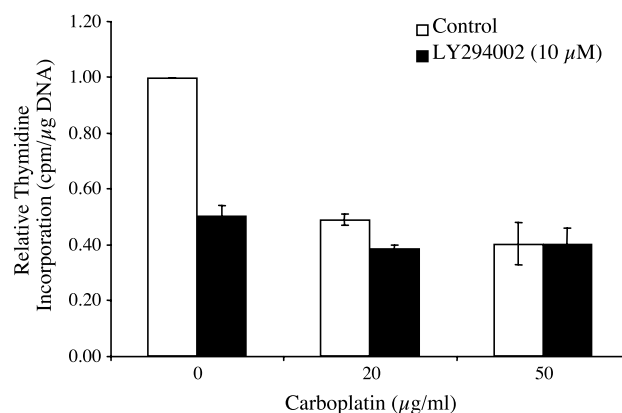


Figure 1. DNA synthesis in cultures of OCC1-SEAP-12 cancer cells following LY294002 and carboplatin treatment. Cell cultures were incubated in the absence or presence of LY294002 (10 mmol/L) and/or carboplatin (0–50 mg/mL) for 18 h. The cultures were pulse-labeled with [^3H]thymidine (5 mCi/well) for 4 h and [^3H]thymidine incorporation into DNA was determined as described in Materials and Methods. The relative [^3H]thymidine incorporation is presented from [^3H]thymidine (counts/min/mg DNA). Columns, mean of four different experiments; bars, SE.

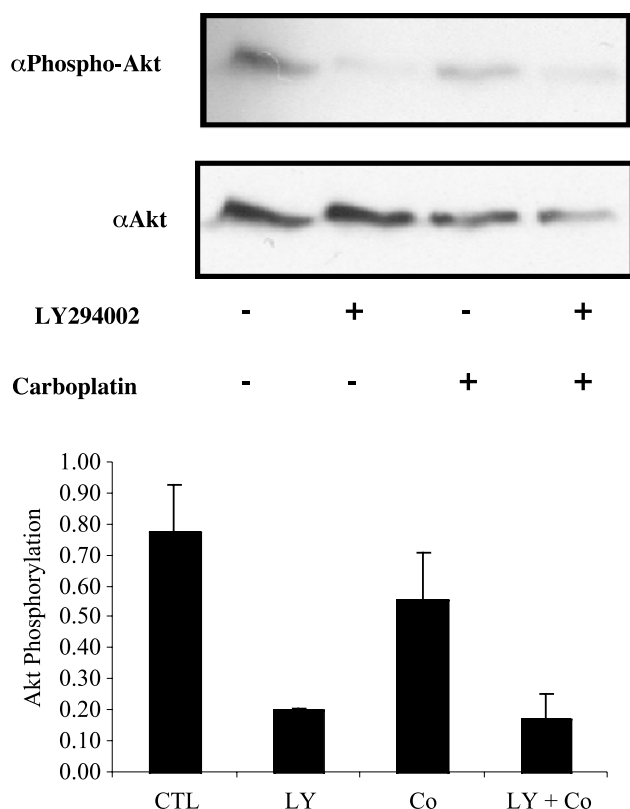


Figure 2. Akt phosphorylation in response to PI3K inhibition in OCC1-SEAP-12 cell cultures. *Top*, cell cultures were incubated in the absence (-) or presence (+) of either LY294002 (10 mmol/L) or carboplatin (50 mg/mL) or a combination of both for 24 h. Aliquots of total cell lysates from cells incubated with treatments were separated by SDS-PAGE and transferred to nylon membranes. Membranes were probed with an antibody to the phosphorylated (α Phospho-Akt) and nonphosphorylated (α Akt) forms of Akt. *Bottom*, densitometry readings of phospho-Akt and Akt Western blots. *Columns*, mean of three separate experiments; *bars*, SE.

Western blot analysis was employed to determine if stimulation of the PI3K pathway was blocked in OCC1-SEAP-12 cell cultures by LY294002. Total cell lysates from OCC1-SEAP-12 cultures treated with LY294002 and/or carboplatin were subjected to SDS-PAGE, transferred to nylon membrane, and probed with an antibody specific to the phosphorylated form of Akt. Basal levels of Akt phosphorylation were observed in lysates from unstimulated OCC1-SEAP-12 cell cultures (Fig. 2). It has been reported that cisplatin treatment stimulates activation of the PI3K pathway (25). Carboplatin had no effect on Akt phosphorylation at either 6 hours (data not shown) or 24 hours (Fig. 2) in OCC1-SEAP-12 cells. The PI3K inhibitor, LY294002, blocked basal levels of Akt phosphorylation at 6 hours (data not shown) and 24 hours (Fig. 2). The inhibition of Akt phosphorylation by LY294002 was not affected by the presence of carboplatin. Inhibition of basal levels of Akt activity correlated with inhibition of basal levels of DNA synthesis.

Cell cultures were assessed for the presence of apoptosis following treatment with either LY294002 or carboplatin.

The DNA in cells undergoing apoptosis is cleaved by endonucleases resulting in DNA fragmentation that can be detected electrophoretically (37). There was no DNA laddering evident in samples from untreated control cultures at either 24 hours (data not shown) or 72 hours (Fig. 3). LY294002 did not induce DNA fragmentation in OCC1-SEAP-12 cells at 24 hours (data not shown) but did so after 72 hours (Fig. 3). DNA laddering was observed in cultures of OCC1-SEAP-12 cells incubated in the presence of carboplatin with and without LY294002 at 48 hours (data not shown) and 72 hours (Fig. 3). An increase in DNA laddering was evident with the combined LY294002 and carboplatin treatment (Fig. 3).

Caspases are proteolytic enzymes that play a central role in the regulation of apoptosis and are activated before apoptotic DNA degradation (17). Caspases are expressed as an inactive precursor and are activated in an amplifying proteolytic cascade (17). Among the caspases, caspase-3 is considered to be a major executioner protease (17). Western blot analysis was used to determine the amount of the activated caspase-3 present. Procaspase-3 is expressed as a 33-kDa protein and is cleaved into 17- and 12-kDa proteolytic products (17). The active 17-kDa caspase-3 was evident at 24 hours in cells treated with the combination of carboplatin and LY294002 (Fig. 4). In contrast, the active form of caspase-3 did not appear until 48 hours in cultures treated with carboplatin alone and not until 72 hours in cultures treated with LY294002 alone (data not shown). There was a concomitant loss of full-length caspase-3 in these samples (Fig. 4).

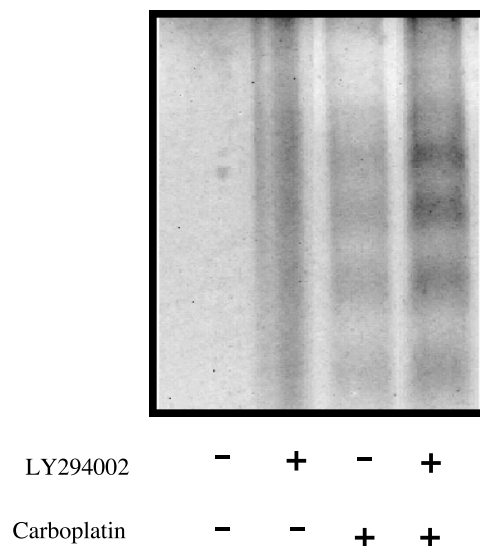


Figure 3. DNA fragmentation in OCC1-SEAP-12 cell cultures in response to LY294002 and carboplatin treatment. OCC1-SEAP-12 cell cultures were incubated in the absence (-) or presence (+) of either LY294002 or carboplatin as well as combined treatments for 72 h. DNA was extracted using a Puregene DNA Isolation kit and separated by electrophoresis on a 1.2% agarose gel. Low molecular weight DNA fragments were visualized with ethidium bromide stain. Representative of three separate experiments.

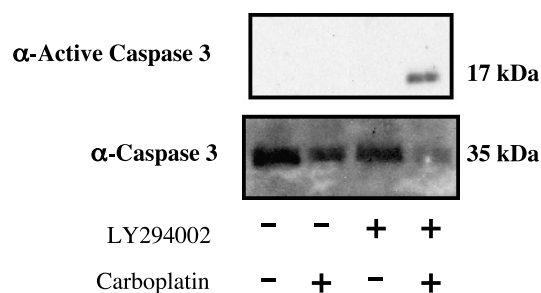


Figure 4. Activation of the proteolytic caspase cascade in response to PI3K inhibition in OCC1-SEAP-12 cell cultures. Cell cultures were incubated in the absence (–) or presence (+) of either LY294002 (10 mmol/L) or carboplatin (50 mg/mL) or a combination of both for 24 h. Aliquots of total cell lysates from cells incubated with treatments were separated by SDS-PAGE and transferred to nylon membranes. Membranes were probed with an antibody to the cleaved (α -Active Caspase 3) and full-length (α -Caspase 3) forms of caspase-3. Representative of three separate experiments.

To determine if the observed increase in apoptosis resulted in a decrease in cell survival, the cell number remaining in culture following prolonged exposure to carboplatin and/or LY294002 was assessed. Levels of DNA in each culture well following 48-hour incubation in the absence or presence of LY294002 with or without carboplatin were used as an indicator of cell number. Doses of 20 and 50 mg/mL carboplatin were used for these experiments as they correlated with *in vivo* doses used. Two doses of LY294002 (5 and 10 mmol/L) were chosen as optimal from the dose-response curve. At these concentrations, LY294002 has been shown to specifically inhibit PI3K and is within the effective dose range shown to inhibit PI3K activity in a variety of cell types. After cells in culture had reached near confluence, treatments were added to culture medium. Following a 48-hour incubation, cell number was reduced by 22% and 44% by 5 and 10 mmol/L LY294002, respectively (Fig. 5). Carboplatin treatment of 20 and 50 mg/mL decreased cell survival by 35% and 54%, respectively (Fig. 5). The greatest reduction in cell number (72%) was seen with the combined treatment of 50 mg/mL carboplatin and 10 mmol/L LY294002 (Fig. 5). There was no significant difference in cell number remaining following the combined treatment of 20 mg/mL carboplatin and 10 mmol/L LY294002 and 50 mg/mL carboplatin alone (Fig. 5). Less than half the amount of carboplatin in combination with LY294002 reduced cell survival to the same extent as the high dose of carboplatin (Fig. 5). Observations indicate that the combined treatment with LY294002 and carboplatin induced optimal apoptosis in the ovarian cancer cells.

In vivo studies were initiated to extend the *in vitro* assessment of the ability of LY294002 to inhibit ovarian cancer cell growth and cell survival. Nude mice were given i.p. injection of OCC1-SEAP-12 cells, and plasma levels of SEAP were used to assay tumor establishment 1 week following OCC1 cell inoculation (36). Mice were then injected with vehicle control, carboplatin, and/or LY294002

every other day for 6 days. The SEAP levels were monitored during and following the treatment regimen. Tumors in mice receiving the combined treatment of LY294002 (50 mg/kg) and carboplatin (60 mg/kg) had a suppressed growth curve when compared with tumors in mice that were treated with vehicle control or LY294002 or carboplatin alone (Fig. 6). Tumors in these mice eventually approached the size of tumors in mice from other treatment groups; however, tumor growth was significantly retarded. In all experiments, we found ascite formation to parallel tumor growth. Mice receiving carboplatin treatment alone exhibited muscle wasting and became anemic. This drug-induced toxicity was not observed with combined treatment. At the point at which tumor burden and ascite formation caused excessive abdominal swelling and/or mice displayed toxic side effects, they were euthanized. Using these variables, mice receiving combined treatment lived much longer. Of the six mice receiving the combined treatment of carboplatin and LY294002 in Fig. 7, four lived 57% longer than the mice in remaining treatment groups and one lived 43% longer. When comparing SEAP levels of the final common bleed for all mice, tumor size was decreased by 2.3-fold as a result of combined treatment of LY294002 and carboplatin in comparison with vehicle control (Fig. 7). There was no significant difference in tumor size among vehicle control, LY294002, and carboplatin treatment groups (Fig. 7). The reduction in tumor burden and ascite formation was also evident in the physical appearance of mice (Fig. 8). Mice from vehicle control, LY294002, and carboplatin treatment groups displayed abdominal swelling that is characteristic of ascite formation and excessive tumor burden (Fig. 8). There was a significant reduction in abdominal swelling in mice treated with the combination of LY294002 and carboplatin (Fig. 8).

During the study, two mice treated with the combined treatment of carboplatin and LY294002 exhibited complete

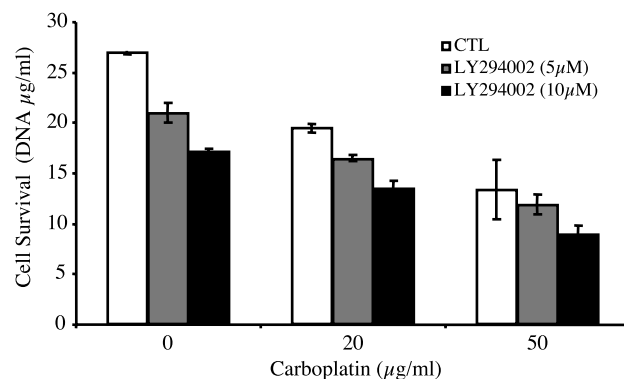


Figure 5. Ovarian tumor cell survival following treatment with carboplatin and a PI3K inhibitor. OCC1-SEAP-12 ovarian cancer cells were incubated for 48 h in the presence or absence of LY294002 (5 and 10 mmol/L) with or without carboplatin (0–50 mg/mL). Cells remaining in the culture wells following the treatment period were suspended in PBS and the amount of DNA present was measured fluorometrically with ethidium bromide (mg DNA/mL) and considered representative of amount of cells surviving in culture. Columns, mean of four different experiments; bars, SE.

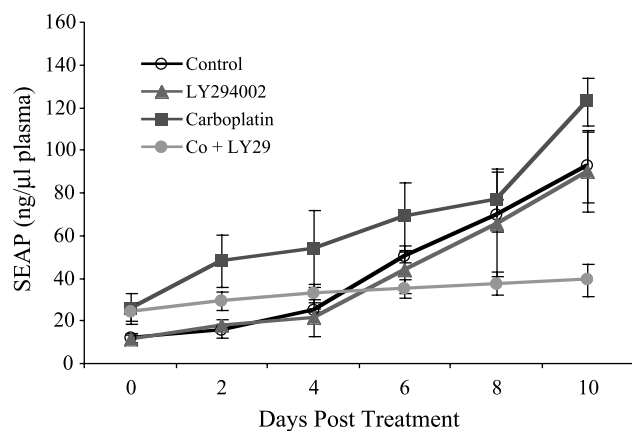


Figure 6. Intraperitoneal tumor progression in response to carboplatin treatment and PI3K inhibition. Nude mice were inoculated with OCC1-SEAP-12 tumor cells by i.p. injection and treatments were initiated within 8 to 10 d. Mice were treated with either vehicle control, carboplatin (60 mg/kg), LY294002 (50 mg/kg), or a combination (Co + LY29) every other day for 6 d. Blood samples were taken at intervals over the treatment period and assayed for SEAP. Points, mean SEAP values from posttreatment tumor growth ($n = 9$ or 10 mice per treatment group); bars, SE. Day 0 is the last day of treatment injection.

remission with no measurable SEAP 30 days after cessation of treatment. These mice were sacrificed at this point and had no observable tumor. However, complete remission was not observed in any other mice ($n = 10$) following carboplatin and LY294002 treatment; therefore, these mice with complete remission were not included in final averages.

Discussion

Athymic mice are a commonly used *in vivo* model in which to study tumorigenesis and assay efficacy of novel chemotherapeutics (33). As ovarian cancers disseminate throughout the peritoneal cavity, the current methods of assaying tumor burden (e.g., measuring weight and volume following tumor dissection or s.c. tumor measurement) can be cumbersome and inaccurate for this type of cancer. Therefore, the current study used an *in vivo* tumor model using a blood marker, which has been developed previously (36). In this model, the level of SEAP directly correlates to tumor burden as evidenced by the observation that SEAP levels increase as a tumor grows (36). A measure of tumor size using a s.c. tumor and correlation measuring SEAP confirmed the use of this new model ($R^2 = 0.92$). Similar correlation was observed with i.p. tumor progression ($R^2 = 0.87$; ref. 36). Therefore, this nude mouse model can be used as an accurate and efficient indicator of tumor burden.

The current observations show that inhibition of the PI3K/Akt pathway results in a decreased proliferation of OCC1-SEAP-12 cells *in vitro*. LY294002 blocked Akt phosphorylation in OCC1-SEAP-12 cultures. The reduction in levels of phosphorylated Akt correlated with the inhibition of proliferation. These and other previous

observations show that the abnormal mitogenic response of cancer cells can be overcome by inhibiting the PI3K/Akt signaling pathway (9, 23, 24, 26). Additionally, the present study corresponds to a recent study by Gao et al. (16) that showed that LY294002 inhibition of PI3K resulted in G₁ cell cycle arrest in ovarian cancer cells, which corresponded to the up-regulation of INK4a expression. Cell cycle progression following exposure to DNA-damaging agents, such as platinum-based compounds, is blocked by p53 activation and subsequent p21^{CIP1/WAF1} expression (27). Despite contrasting mechanisms, LY294002 and carboplatin were equally effective, but not additive, in blocking ovarian cancer cell proliferation in these studies.

In addition to the attenuation of OCC1-SEAP-12 cell proliferation, a decrease in cell survival was seen following PI3K inhibition. However, compared with the growth response, LY294002 alone was not as effective in promoting apoptosis as carboplatin. The combination of both compounds was additive as indicated by a marked enhancement of DNA laddering in cells following the combined treatment of carboplatin and LY294002. Furthermore, activation of caspase-3 was induced at a much earlier time point with the combined treatment. The active or cleaved form of caspase-3 was evident within 24 hours following combined treatment and was not detectable until 48 hours following carboplatin treatment alone or at 72 hours following LY294002 treatment alone. In addition, a significantly lower dose of carboplatin was needed to reduce cell number in culture when in the presence of LY294002. Observations suggest that inhibition of the PI3K/Akt pathway can sensitize ovarian cancer cells to the toxic effects of carboplatin.

Most importantly, the current study shows that LY294002 in combination with carboplatin was effective in inhibiting

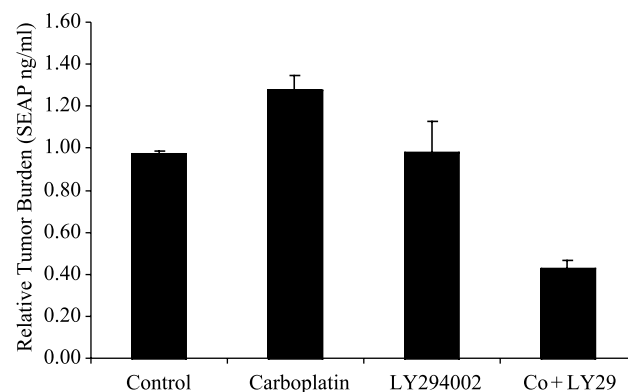


Figure 7. Effects of LY294002 and carboplatin on tumor growth in mice inoculated with OCC1-SEAP-12 ovarian cancer cells. Nude mice were inoculated with OCC1-SEAP-12 tumor cells by i.p. injection and treatments were initiated within 8 to 10 d. Mice were treated with either vehicle control, carboplatin (60 mg/kg), LY294002 (50 mg/kg), or combination every other day for 6 d. Blood samples were taken at intervals over the treatment period and assayed for SEAP (ng SEAP/mL plasma). Columns, mean relative final tumor burden (as measured by plasma levels of SEAP) at last common bleed ($n = 9$ or 10 mice per group); bars, SE.

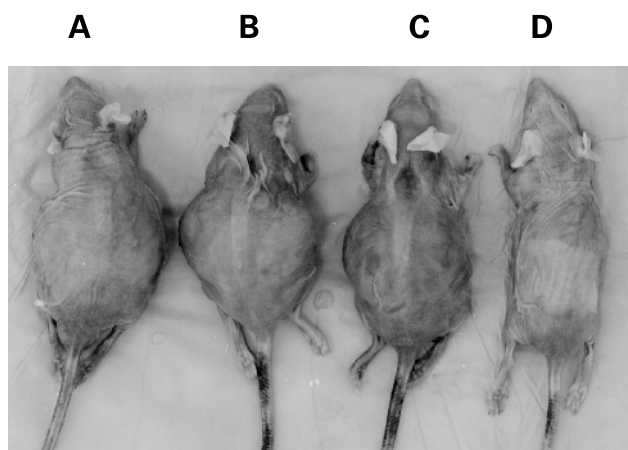


Figure 8. Appearance of mice after treatment with LY294002 and carboplatin alone and in combination. Four representative nude mice inoculated with OCC1-SEAP-12 cells and treated with either vehicle control (PBS + DMSO) only (A), carboplatin (60 mg/kg) alone (B), LY294002 (50 mg/kg) alone (C), or carboplatin plus LY294002 (D). Treatments were given every other day for 6 d. Mice were euthanized ~ 14 d following cessation of treatment.

ovarian cancer cell xenograft growth in a nude mouse model. There was a significant delay in growth of ovarian tumors in mice receiving both carboplatin and LY294002 compared with other treatment groups. This resulted in a significant increase in survival rate when compared with all other treatment groups. The current study showed that LY294002 alone was not effective in inhibiting tumor progression, as was observed in a previous study by Hu et al. (26, 29). This is most likely a result of use of a dose of 50 mg/kg LY294002 versus a dose of 100 mg/kg, which was found to be most effective in the experiments done by Hu et al. (29). In addition, the number of injections was fewer and the duration of treatment was shorter (every other day for 5 days versus 3 days a week for 4 weeks). The current study focused on the potential use of PI3K inhibitor as an adjunct chemotherapy with carboplatin.

The current study used a single cell line, OCC1. Although this ovarian tumor cell line is partially chemoresistant, the data are based on only one cell line. Therefore, generalization to all ovarian tumors cannot be made and will require further analysis of other cell lines and primary ovarian tumor cells. The observations support the potential that inhibition of PI3K may be useful as an adjunct chemotherapy, but analysis of a wider variety of cells is now needed. The heterogeneity of tumor cell populations within a primary tumor needs to be considered regarding the actions of any chemotherapy. Although a therapy such as carboplatin may eliminate those chemosensitive cells within a tumor, the recurrence of the tumor with the chemoresistant cell populations is the challenge of any chemotherapy. If an adjunct therapy, such as the PI3K inhibitor, can increase the percentage of cells sensitive to a chemotherapy, then more effective treatment and reduced rates of recurrence can be obtained. Critical signal

transduction pathways, such as the PI3K pathway, that block cellular apoptosis and modulate cell growth are often involved in the mechanisms of chemoresistance. Therefore, inhibiting these types of signal transduction pathways will facilitate chemotherapy treatments. Although further studies are required on a wider variety of ovarian tumors and cell lines, the current study suggests that inhibitors of PI3K will be useful as potential adjunct chemotherapy treatments.

Observations suggest that the combined treatment of carboplatin and LY294002 can inhibit ovarian tumor progression and support the use of the PI3K inhibitor, LY294002, with the platinum-based drug therapy as an appropriate treatment course for ovarian cancer. The compound LY294002 is toxic when given systemically; therefore, its administration would have to be through i.p. infusion. Ovarian cancer generally remains confined to the abdominal cavity, and as a result, i.p. infusion of therapy is being pursued as an appropriate delivery system for this disease. Therefore, this mode of delivery would make LY294002 a plausible alternative chemotherapy. Future studies are needed to evaluate the potential of LY294002 as an effective adjunct chemotherapy against later-stage or more established tumors. Furthermore, it still remains to be determined if LY294002 administration would enable the use of a lower dose of carboplatin. If the use of LY294002 as an adjunctive chemotherapy would increase the efficacy of lower doses of carboplatin, the combination may be an effective way to control tumor growth in ovarian cancer patients with acceptable side effects.

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