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Inhibition of Phosphatidylinositol 3′-Kinase Increases Efficacy of Paclitaxel in *in Vitro* and *in Vivo* Ovarian Cancer Models[1]

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ABSTRACT

Phosphatidylinositol 3′-kinase (PI3k) is implicated in a wide array of biological and pathophysiological responses. Thus, inhibiting molecules involved in its signal transduction pathway is a possible means of treating cancer. Our previous studies demonstrated that LY294002, a potent and selective PI3k inhibitor, decreases growth of ovarian carcinoma and ascites formation in an athymic mouse xenogeneic transplant model of ovarian cancer. However, the dose of LY294002 used to decrease tumor growth resulted in significant dermatological toxicity. We demonstrate herein that introduction of an active catalytic subunit of PI3k into an ovarian cancer cell line, and thus activation of the PI3k/AKT pathway, confers resistance to the effects of paclitaxel, one of the major drugs used in ovarian cancer therapy. The resistance to paclitaxel can be reversed *in vitro* by inhibition of PI3k. Therefore, we evaluated whether combined therapy with paclitaxel and LY294002 would result in increased efficacy and allow utilization of doses of LY294002 that do not induce dermatological toxicity. Two weeks after i.p. inoculation with OVCAR-3 ovarian cancer cells, mice were treated i.p. with LY294002 plus paclitaxel, each three times weekly on alternate days, for 4 weeks. Tumor burden in the LY294002 + paclitaxel, LY 294002 alone, and paclitaxel alone groups was reduced by 80% ($P < 0.01$), 38% ($P < 0.05$), and 51% ($P < 0.05$), respectively, compared with controls. Virtually no ascites developed in the combined treatment group; mean volume of ascites in the controls was 3.7 ml. Treatment with LY294002 alone reduced ascites by 70% ($P < 0.01$), whereas paclitaxel alone reduced ascites slightly but not significantly. No dermatological lesions or weight loss were observed in any treatment group. *In vivo* and *in vitro* morphological studies demonstrated that inhibition of PI3k enhanced paclitaxel-induced apoptosis in the human ovarian cancers. Our data suggest that a combination of a PI3k inhibitor and conventional chemotherapy may provide an effective approach to inhibiting tumor growth and ascites production in ovarian cancer with acceptable side effects.

INTRODUCTION

Aberrant function of PI3k[3] contributes to mitogenesis, cellular growth, and cellular transformation in a variety of neoplasms including ovarian cancer (1–3). PI3k is a cytosolic enzyme consisting of an M_r 85,000 regulatory subunit and a M_r 110,000 catalytic subunit (4). *PIK3CA*, the gene encoding the P110 α catalytic subunit of PI3k, is present in increased copy number in primary ovarian cancer cells and several ovarian epithelial cancer cell lines (5). Furthermore, AKT2 is overexpressed in a substantial number of ovarian cancers (6), and PTEN protein levels may be decreased in some of these tumors (7). This results in increased signaling through this pathway as indicated by phosphorylation of AKT *in vitro* and *in vivo* (8). Thus, inhibition of PI3k or molecules involved in the PI3k signaling pathway is an attractive approach to therapy for this disease. We have demonstrated that LY294002, a potent and selective inhibitor of PI3k, decreases growth of ovarian carcinoma and ascites formation in an athymic mouse model of i.p. ovarian cancer (9). Mean tumor burden in the LY294002-treated group was reduced by ~65% *versus* controls. However, 80% of the treated mice developed dry, scaly skin. Three days after cessation of LY294002, which is a reversible inhibitor of PI3k, the skin returned to a normal appearance. These observations imply that the side effects appearing in the LY294002-treated mice are largely dependent on the dose and/or frequency of LY294002 treatment, both of which might be reduced to avoid skin toxicity.

Paclitaxel promotes assembly of microtubules, inhibits tubulin disassembly and DNA synthesis (10, 11), and causes apoptotic cell death in a variety of cancer cell types (12). Although paclitaxel is an important chemotherapeutic agent for treatment of patients with ovarian epithelial cancer, with high initial response rates, the majority of patients with advanced disease eventually develop resistance (13). Several studies have recently indicated that alterations in the PI3k/AKT signal transduction pathway can modulate sensitivity to cancer chemotherapy (14–16). Overexpression of an activated AKT decreases paclitaxel-induced apoptosis in ovarian cancer cells (14). BAD, a downstream target of the PI3k/AKT pathway, has been demonstrated to partially reverse paclitaxel resistance in ovarian cancer cells (17). Furthermore, overexpression of BAD increases sensitivity to paclitaxel (17). Inhibition of PI3k by LY294002 or wortmannin, effective PI3k inhibitors, enhances gemcitabine-induced apoptosis in human pancreatic cancer cells (15). Wortmannin also sensitizes cells to the effects of bleomycin (16). The PI3k pathway contributes to cell survival by multiple mechanisms, including phosphorylation of BAD, caspase 9, GSK3, p21, p27, and p70S6K, all of which can regulate apoptosis (17–19). We demonstrate that overexpression of an activated PI3k decreases sensitivity to paclitaxel, a process that is reversed by inhibition of PI3k with LY294002. Thus, we elected to assess the ability of LY294002 to sensitize cells to paclitaxel, allowing us to assess a lower dosage of LY294002. We also demonstrate that a combination of a PI3k inhibitor with a chemotherapeutic agent is an effective means of controlling the growth of ovarian carcinoma with acceptable side effects.

MATERIALS AND METHODS

Materials

LY294002, a PI3k inhibitor, was a generous gift from Dr. J. Starling, Eli Lilly and Company (Indianapolis, IN). Paclitaxel was obtained from Sigma Chemical Co. (St. Louis, MO). The human OVCAR-3 cell line, which causes ascites earlier in the course of the disease process than the SKOV3 cell line that we have used previously (20), was kindly provided by Dr. T. Hamilton, Fox Chase Cancer Center, Philadelphia, PA. Dov13, which is the only ovarian cancer cell line that we have identified with a normal PI3k pathway, was obtained from its developer, Dr. R. Bast (Houston, Texas). Glass slides were obtained from Lab-Tek, Nalge Nunc International (Naperville, IL). Cell culture reagents were obtained from the Cell Culture Facility, UCSF.

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[3] The abbreviations used are: PI3k, phosphatidylinositol 3′-kinase; UCSF, University of California, San Francisco; BW, body weight; FBS, fetal bovine serum; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling.

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In Vivo Studies

Experimental Animals. Four groups of female athymic immunodeficient mice (Simonsen Laboratories, Gilroy, CA) were delivered to the UCSF Animal Care Facility, housed in isolated conditions, fed autoclaved standard pellets and water, and allowed to adapt to their new environment. All of the protocols involving immunodeficient mice were approved by the Committee on Animal Care, UCSF.

Experimental Design. Four groups of female athymic nude mice (5–7 weeks of age) were inoculated i.p. with OVCAR-3 cells. Two weeks after inoculation, one group of mice ($n = 12$) was treated with LY294002 (100 mg/kg BW) three times weekly plus paclitaxel (20 mg/kg BW) three times weekly, on alternate days, for 4 weeks. A second group of mice ($n = 12$; two groups of six each) was treated with LY294002 alone (100 mg/kg BW) three times weekly. The third group ($n = 12$; two groups of 6 each) was treated with paclitaxel alone (20 mg/kg BW). The remainder of the mice ($n = 12$; two groups of six each) received vehicle (DMSO + PBS) alone. The dose of paclitaxel (20 mg/kg BW) was based on previous studies (21, 22).

Methods. To prepare cells for inoculation, they were collected from the ascites fluid of athymic mice inoculated previously with OVCAR-3 cells. Ascites fluid was collected and placed in a 4°C refrigerator for 1–2 h. The supernatant was then discarded. The cells were diluted with RPMI 1640 supplemented with 2.0 g/liter glucose and 0.3 g/liter L-glutamine, which had been prewarmed in a 37°C incubator. Athymic nude mice (5–7 weeks of age; $n = 48$) were inoculated i.p. with OVCAR-3 cells, 2×10^6 cells/mouse in 500 μl of RPMI 1640. Abdominal circumference and BW were measured twice weekly. At the end of the experiment, mice underwent euthanasia with CO_2. The volume of ascites was measured, tumor tissue was excised, weighed, fixed in 4% paraformaldehyde, and embedded in paraffin. Paraffin sections (5 μm) were used for histochemical analysis.

In Vitro Studies

OVCAR-3 Cell Cultures. OVCAR-3 cells were seeded (5×10^4) on eight-well glass slides. The cells were cultured in RPMI 1640 containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a 37°C incubator in a humidified atmosphere of 95% O_2/5% CO_2. After the cells were seeded (24 h), medium was removed and replaced with culture medium in the presence of paclitaxel (10 μM) for 48 h, or with the same concentration of paclitaxel for 24 h followed by LY294002 (5 μM) for an additional 24 h or 5 μM LY294002 alone for 24 h. The concentration and duration of paclitaxel or LY294002 treatment were chosen based on preliminary studies examining their effects on induction of apoptosis. Slides were prepared for morphological studies by fixing with 2% paraformaldehyde.

DOV13 Cell Transfection. DOV13 Mp110* is a cell clone expressing constitutively activated PI3k p110α catalytic subunit. A constitutively activated form of PI3k p110α catalytic subunit (myristylated and myc-epitope-tagged murine p110α with iSH2 domain of p85 subunit attached to its NH_2 terminus) in the pSRα vector was provided by Dr. Anke Klippel (Atugen AG Berlin, Germany) (23). The Dov13 ovarian cancer cell line was cultured in RPMI 1640 supplemented with 10% FBS. Cells were cotransfected using Fugene 6 Transfection Reagent as recommended by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN) with the expression vector containing constitutively activated PI3k p110α cDNA and a pCDNA3 vector carrying the *G418* resistance gene. After transfection, cells were selected by culture in G418 (400 μg/ml). Surviving cells were cloned and expanded. Surviving clones were screened by immunoblotting for the presence of epitope-tagged PI3kp110α. Stably transfected cells are designated DOV13 Mp110*.

DOV13 Cell Cultures. DOV13 cells were pulse-treated with different concentrations of paclitaxel (0–1 μM) for 2 h as indicated. Cells were washed and continued in culture in RPMI 1640 supplemented with 10% FBS for a total 24-h incubation. In the case of PI3k inhibition, cells were incubated with LY294002 for 15 min before paclitaxel treatment for 2 h. Paclitaxel was removed by washing, and the cells were cultured for an additional 22 h in medium containing LY294002 (thus, LY 294002 was present throughout the culture). After treatment, cells were harvested for assessment of apoptosis.

Assessment of Apoptosis

Paraffin sections (5 μm) of ovarian cancer tissue from OVCAR-3 cell-inoculated mice treated with LY294002 + paclitaxel or slides from OVCAR-3 cells cultured with LY294002 + paclitaxel were used to assess apoptosis. DNA labeling with digoxigenin dUTP and terminal transferase, followed by immunocytochemical staining with peroxidase-coupled antidigoxigenin antibody and diaminobenzidine, were carried out with the reagents supplied in the Apoptag kit (Intergen, Purchase, NY) according to the manufacturer's instructions, except that Tris (tris[hydroxymethyl] amino-methane and tris[hydroxymethyl] amino-methane hydrochloride) was substituted for phosphate in the wash buffer. After light counterstaining with hematoxylin, nuclei that stained brown were scored as positive for apoptosis and those that stained blue were scored as negative. At least five ×300 microscopic fields were scored, and the apoptotic index was calculated as the percentage of cells that were scored positive. The same Apoptag kit was used for determination of cell apoptosis in OVCAR-3 cells from the *in vitro* study.

Apoptosis in DOV13 cells was determined by measuring the presence of cytoplasmic histone-associated DNA fragments using an ELISA-based assay (Roche Molecular Biochemicals). TUNEL-positive cells (apoptotic cells) were measured using Apo-BrDU kit as recommended by the manufacturer (Phoenix Flow Systems, Inc., San Diego, CA) to label DNA breaks. The number of cells with DNA breaks was analyzed by flow cytometry.

Light Microscopy and Analysis

Tissue sections of ovarian cancer from OVCAR-3 cell-inoculated mice treated with LY294002 + paclitaxel were examined with a Leica DMRB or Leica Ortholux II photomicroscope at low and high magnifications. Images were collected with a Photonics DEI-470 CCD camera and a RasterOps 24XLTV frame-grabber, imported directly into Adobe PhotoShop 4.0, and stored on a ZIP external 100 MB drive (Iomega). Photomicrographic plates were composed from the original data in PhotoShop, without alteration or manipulation, and annotated with rub-on letters and symbols.

Statistics

Results are presented as means ± SE. Data were analyzed using one-way ANOVA followed by an unpaired Student's t test for comparison between groups. Differences between groups were considered statistically significant at $P < 0.05$. Experiments *in vivo* were performed in duplicate, whereas experiments *in vitro* were performed in triplicate.

RESULTS

We have demonstrated previously that PI3k is frequently amplified at the genomic, mRNA, and protein levels in ovarian cancers (5). To assess whether this amplification could alter sensitivity to paclitaxel, we introduced an activated and membrane-targeted PI3k catalytic subunit (myrp110α*; ×4) into DOV13 cells, which do not overexpress PI3k (5). Modest expression of the activated PI3k (Fig. 1) resulted in a marked decrease in the apoptosis induced by paclitaxel over a concentration range from 30 to 1000 nM (Fig. 1). As indicated in Table 1, the resistance to paclitaxel, conferred by activated PI3k, was reversed by incubation of the myrp110α*-transfected DOV13 with LY294002. Thus, activation of PI3k increases paclitaxel resistance, a process which is abrogated by inhibition of PI3k. This suggests that the combination of paclitaxel and LY294002 may be effective *in vivo* and also decrease tumor growth and ascites formation without concomitant toxicity. Unfortunately, DOV13 cells do not grow *in vivo* and all of the ovarian cancer cell lines that do grow *in vivo* have activated PI3k, precluding introduction of activated PI3k into these cells. Thus, to test this hypothesis, we used OVCAR-3 cells, which have increased PI3k levels and activity and are, thus, sensitive to the effects of LY294002 (5, 6).

We examined the effects of the PI3k inhibitor LY294002 and paclitaxel, singly and in combination, on the control of ovarian tumor growth and ascites formation to assess whether combination therapy

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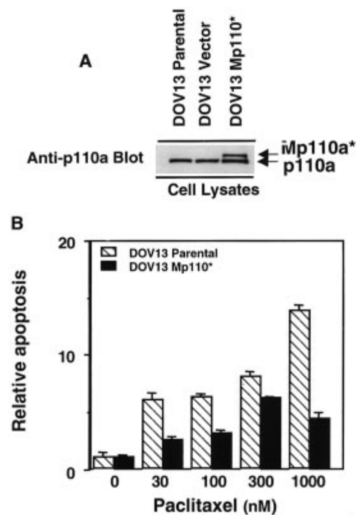

Fig. 1. Expression of activated PI3k p110 in DOV13 cells induces resistance to paclitaxel. *A,* DOV13 cells were stably transfected with control vector or expression vector carrying activated PI3k p110α cDNA. Cells were lysed and cellular proteins resolved in 8% SDS PAGE and immunoblotted by rabbit anti-PI3k p110α antibody. *B,* parental DOV13 or DOV13 Mp110* cells were pulse-treated with different concentrations of paclitaxel for 2 h as indicated. Cells were then cultured in culture in RPMI 1640 supplemented with 10% FBS for an additional 22 h. Cells were lysed, and cellular protein was subjected to an ELISA-based assay to measure the presence of cytoplasmic histone-associated DNA fragments. Relative apoptosis was calculated as A_{405nm} from paclitaxel-treated cells/A_{405nm} from untreated cells. The data are presented as mean from triplicates at each treatment dose; *bars,* ± SD.

Table 1 *Inhibition of PI3k in DOV13Mp110* cells increases paclitaxel-induced apoptosis*

Parental DOV13 or DOV13 Mp110* cells were treated with 1 mM paclitaxel in the absence or presence of 20 mM LY294002 as described in "Materials and Methods." Cells were harvested, and the TUNEL/positive cells (apoptotic cells) were measured using an Apo-BrDU kit to label DNA breaks. The data were analyzed by flow cytometry and are presented as % of TUNEL/positive cells in the total cell population. Fold increase was calculated as number of apoptotic cells after LY294002/number of apoptotic cells after 0.2% DMSO treatment cells and number of apoptotic after paclitaxel treatment/number of apoptotic cells in control medium.

	DOV13 parental cell line		DOV13Mp110* cell line	
Treatment	TUNEL/positive cells (%)	(Fold increase)	TUNEL/positive cells (%)	(Fold increase)
Control	1.5		0.9	
0.2% DMSO	2.9		1.5	
LY294002 (20 mM)	4.9	(1.7)	3.7	(2.5)
Paclitaxel (1 mM)	4.7	(3.1)	0.8	(2.9)
Paclitaxel + LY294002	7.3	(2.5)	3.2	(2.1)

would increase the therapeutic efficacy of each agent and also reduce deleterious side effects. We used a homotypic model of i.p. ovarian carcinoma in athymic immunodeficient mice (20), which was used previously in our laboratory to assess effects of LY294002 on tumor growth (9). Two animals in the paclitaxel + LY294002 treatment group ($n = 12$) died, one 3 days and the other 14 days after initiation of treatment. One animal in the paclitaxel-only treatment group ($n = 12$) died 2 weeks after initiation of treatment. The reason for these deaths is unknown.

At postmortem examination (4 weeks after initiation of therapy), tumors were found on the surface of the peritoneum, intestines, omentum, and uterus in both treatment and control groups. The extent of tumor involvement of the diaphragm and/or the hilus of the liver varied among the different treatment groups. Seventy five, 25, and 18% of the mice in the control, LY294002-treated, and paclitaxel-treated groups, respectively, had tumors on the diaphragm and in the hilus of the liver. Strikingly, combined LY294002 + paclitaxel treatment abrogated diaphragmatic and hilar tumors.

Fig. 2 illustrates four representative mice treated with vehicle (PBS + DMSO) only (mouse A); with LY294002 alone (mouse B); with LY294002 + paclitaxel (mouse C); and with paclitaxel alone (mouse D). Mouse A had significant abdominal swelling, whereas swelling was much less marked in mice treated with LY294002 with and without paclitaxel.

In our previous study using LY294002 at a higher total dose (100mg/kg daily; Ref. 9), the majority of the mice developed a dry patchy dermatitis, which resolved after cessation of the drug. In the present study, alternate day dosing of LY294002 (100mg/kg daily, 1/2 the total dose in the previous study) in the presence or absence of paclitaxel did not induce dermatological changes. Thus, decreasing the dose of LY294002 by a factor of two was sufficient to decrease the toxicity.

Control of Ovarian Tumor Growth. Results of the combination treatment of LY294002 + paclitaxel on tumor growth are shown in Fig. 3*A*. Mean tumor burden in the combined LY294002 + paclitaxel-treated group (0.526 ± 0.11 g) was significantly ($P < 0.01$) less than that of the controls (2.64 ± 0.36 g). Tumor burden in the LY294002 + paclitaxel, LY 294002 alone, and paclitaxel alone groups was reduced by 80.0% ($P < 0.01$), 38.2% ($P < 0.05$), and 51.7% ($P < 0.05$), respectively, compared with the control group.

Control of Ascites Formation. Fig. 3*B* shows the results of the combined treatment with LY294002 + paclitaxel on ascites formation. The mean volume of ascites in the control group was 3.7 ml. In contrast, virtually no ascites developed in the LY294002 + paclitaxel-treated group. LY294002 alone significantly ($P < 0.01$) reduced ascites formation by 70.7% (1.1 ml), whereas paclitaxel reduced ascites formation slightly but not significantly. The difference in ascites reduction between LY294002 alone and LY294002 + paclitaxel was not statistically significant.

Apoptosis. We demonstrated apoptosis in ovarian cancer tissue from OVCAR-3-inoculated mice; we found brown-staining Apoptag-positive nuclei, indicative of cells undergoing apoptosis. Apoptosis in OVCAR-3 cells in the LY294002-treated group and paclitaxel-treated groups was ~20% and 30%, respectively, of the total cell population. More than 60% of the cells in the field of vision (×300) on the tissue slides from the LY294002 + paclitaxel group were necrotic or had undergone apoptosis with cytoplasmic debris and calcification. At least five ×300 microscopic fields were scored. There were no significant changes in control mice.

We also demonstrated apoptosis of cultured OVCAR-3 cells after 48-h treatment with 10 μM of paclitaxel or with the same concentration of paclitaxel for 24 h, after which 5 μM of LY294002 was administered for an additional 24 h or cells were exposed to 5 μM of LY294002 alone for 24 h. Again, we observed Apoptag-positive cells.

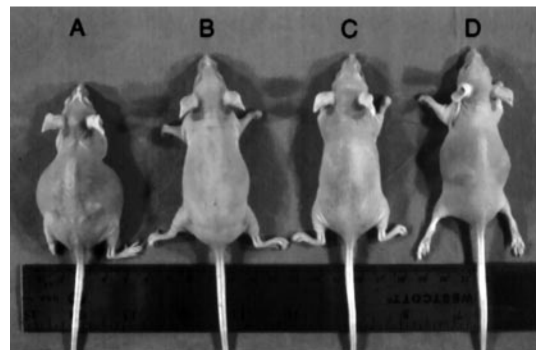

Fig. 2. Appearance of mice after treatment with LY294002 and paclitaxel alone and in combination. Four representative athymic mice inoculated with OVCAR-3 cells treated i.p. with vehicle (PBS + DMSO) only (*A*); with LY294002 alone (*B*); with LY294002 + paclitaxel (*C*); or with paclitaxel alone (*D*).

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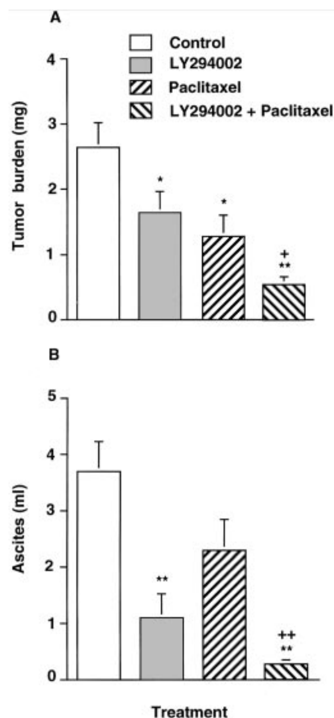

Fig. 3. Effects of LY294002 plus paclitaxel on i.p. tumor growth and ascites formation in mice inoculated with OVCAR-3 cells. Treatments were started 2 weeks after inoculation. Treatment groups consist of control (vehicle alone), LY294002 alone, LY294002 + paclitaxel, and paclitaxel alone. At the end of the experiment (4 weeks of treatment), mice were sacrificed. At autopsy, tumors were excised and weighed, and ascites fluid was collected and measured. *A,* effects of LY294002 and paclitaxel on tumor growth (n = 24); *B,* effects of LY294002 and paclitaxel on ascites formation (n = 24). Results are from two independent experiments. Data are expressed as the mean; *bars,* ± SD. **, $P < 0.01$ *versus* control; +, $P < 0.05$ *versus* paclitaxel; ++, $P < 0.01$ *versus* paclitaxel.

In combined treatment with paclitaxel + LY294002, ~50% of the treated cells were apoptotic. However, only ~10% of OVCAR-3 cells were apoptotic in the paclitaxel-treated group, and <10% of OVCAR-3 cells were apoptotic in the group treated with LY294002.

DISCUSSION

Ovarian cancer is the fifth most common cause of death from cancer in American women and the most lethal of the gynecological malignancies (24). Although there has been some improvement in survival duration with the introduction of paclitaxel and cisplatin therapy, the mortality rates in women with advanced, recurrent, or persistent ovarian cancer have remained largely unchanged for the last 4 decades (24). Our previous study indicated that the PI3k inhibitor LY294002 significantly decreased ovarian cancer growth and ascites formation in athymic mice inoculated with OVCAR-3 cells *in vivo* and reduced OVCAR-3 cell proliferation *in vitro* (9). However, 80% of the treated mice developed dry, scaly skin, which may have been dose-, frequency-, and/or duration-dependent. In the present study, we reduced the frequency to every second day and, therefore, halved the total dose of LY294002 to attempt to avoid these dermatological effects. At this lower dose, none of the mice treated with LY294002 developed dermatological lesions.

Our data demonstrate that the combination of paclitaxel + LY294002 causes a marked inhibition (>80%; $P < 0.01$) of tumor growth and spread, in addition to virtually total inhibition of ascites production ($P < 0.05$), whereas paclitaxel alone reduced tumor burden 51% ($P < 0.01$), and LY294002 caused a 38% ($P < 0.05$) reduction in tumor burden and 70% ($P < 0.01$) inhibition of ascites formation. The effect of the combined therapy on growth and tumor dissemination was markedly greater than those of either agent alone. These results suggest that combining an inhibitor of PI3k with a chemotherapeutic agent such as paclitaxel is an effective way to control the growth of ovarian carcinoma with fewer side effects than either agent alone. This conclusion is supported by the observation that introduction of an activated PI3k into ovarian cancer cells, which do not have increased levels of PI3k-induced resistance to paclitaxel, a process that was reversed by the inhibition of PI3k (Fig. 1; Table 1). Because ~40% of ovarian cancers have abnormalities in the PI3k pathway (5), ovarian cancer may be a particularly appropriate target for therapy with inhibitors of the PI3k pathway. Abnormalities of the PI3k pathway are also found in a variety of other neoplasms (25), suggesting that this approach may be effective in a number of other malignancies.

The dose and frequency of LY294002 used in our previous study in which dermatological lesions developed was 100 mg/kg BW daily for 3 weeks. In the present study, we used the same dose but reduced the frequency to 3 days weekly on alternate days for 4 weeks. None of the LY294002-treated mice developed skin changes. The reasons that lower frequency and, therefore, lower total weekly dose of LY294002 administration eliminated the dermatological effects of LY294002 alone are still unclear. Dry and scaly skin is an indicator of hyperkeratosis (26), and keratinization can be seen subsequent to apoptosis (27). *In vivo*, apoptotic cells undergo efficient phagocytosis by macrophages, a process thought to be essential for tissue remodeling (28). PI3k is required for phagocytosis of apoptotic cells, and LY294002 inhibits the phagocytic process (29). In the present study, the lower total dose as a consequence of decreased frequency of administration of LY294002 may have allowed apoptotic cells to undergo efficient phagocytosis by macrophages, preventing the dermatological lesions.

The most important finding in the present study is that inhibition of PI3k by LY294002 enhanced paclitaxel-induced apoptosis in athymic mice inoculated with cells from a human ovarian cancer cell line as well as ovarian cancer cells cultured *in vitro*. A previous study demonstrated that OVCAR-3 cells, which exhibit endogenous amplification of AKT2, were indeed more resistant to paclitaxel than CAOV-3 ovarian cancer cells, which only express low levels of AKT2 (30). These findings suggest that alteration of the PI3k/AKT signal transduction pathway may alter drug sensitivity of ovarian cancer cells. We confirmed that this was because of increased activity of the PI3k pathway by demonstrating that introduction of an activated PI3k catalytic subunit into Dov13 cells (Fig. 1; Table 1) rendered cells resistant to paclitaxel, a process that was reversed by incubation with LY294002. The mechanism by which LY294002 enhances paclitaxel-induced apoptosis is still unclear; however, it clearly involves signaling through the PI3k pathway. Apoptosis is a process of regulated cell death characterized by cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation (31). A wild-type p53 protein often is involved in the nuclear events mediating apoptosis (32, 33). Mutational inactivation of p53 can inhibit apoptosis and contribute to the resistance of tumor cells to chemotherapeutic agents and radiation (33). However, p53 is undetectable in OVCAR-3 cells (34), the ovarian cancer cell line used in the present study. Thus, the results suggest that the apoptosis that occurs in OVCAR-3 cells is independent of p53.

Recent observations indicate that the gene encoding the P110 α catalytic subunit of PI3k is increased in copy number in ~40% of primary ovarian cancer cells (5). Inhibitors of this pathway reduce growth of ovarian cancer cell lines, including OVCAR-3, *in vitro* (5, 9) and *in vivo*. (9). OVCAR-3 cells and many primary ovarian cancers also exhibit endogenous amplification of AKT2, a downstream signaling molecule in the PI3k pathway (35). AKT can translocate to the nucleus where it may contribute to the regulation of the transcription

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of genes mediating cell survival (36, 37). One possible mechanism by which AKT functions to promote survival is through the phosphorylation of BAD (38, 39), which results in inactivation of apoptotic function (39). BAD, a proapoptotic member of the Bcl-2 family (40), promotes apoptosis by binding and blocking the activity of Bcl-XL, a cell survival factor. The Bcl-2 family of proteins has been implicated in cell survival decisions (19, 40). Several Bcl-2 family proteins act to regulate the release of cytochrome *c* from mitochondria, a key event in the process of apoptosis (41). BAD may prevent or reverse resistance to chemotherapeutic agents such as paclitaxel (17). Elevated levels of activated PKB/AKT can protect cells from undergoing apoptosis induced by cytotoxic drugs and contribute to drug resistance by phosphorylating BAD, resulting in its binding to 14-3-3 and sequestration away from BCLX (15, 42). Ovarian cancer cells overexpressing AKT1 or having *AKT2* gene amplification are more resistant to paclitaxel treatment than parental cells (14). Overexpression of AKT can lead to phosphorylation of BAD and prevent it from binding to Bcl-XL and inhibiting the release of cytochrome *c* induced by paclitaxel (14). In addition, AKT directly inhibits cell death by preventing the release of cytochrome *c* from mitochondria (43). There is evidence that LY294002 blocks PKB/AKT phosphorylation, and the reduction of phosphorylated PKB/AKT levels correlated with enhancement of gemcitabine-induced apoptosis in human pancreatic cancer cells (15). The loss of mitochondrial inner membrane potential appears to be a common event during apoptosis in mammalian cells (44). LY294002 significantly enhanced the loss of mitochondrial inner membrane potential in human pancreatic cancer cells exposed to gemcitabine. In the present study, LY294002-enhanced, paclitaxel-induced apoptosis may have involved the AKT/BAD pathway.

The present study is consistent with our previous report indicating that LY294002 markedly inhibits the ascites formation often associated with ovarian carcinoma (9). In that earlier report, we noted that, as vascular endothelial growth factor/vascular permeability factor induces the processes leading to endothelial cell survival through the PI3k/AKT signal transduction pathway (45), LY294002, by inhibiting PI3k activity, may have blocked the signal transduction pathway of vascular endothelial growth factor/vascular permeability factor. This, in turn, may have inhibited the ascites formation associated with ovarian carcinoma. Our present study also has shown that combination treatment with LY294002 + paclitaxel reduces ascites more extensively than treatment with paclitaxel alone and suggests the effectiveness of a PI3k inhibitor plus paclitaxel in reduction of ascites.

In summary, our data demonstrate the enhancing effects of LY294002 on paclitaxel in reducing tumor burden and ascites formation in athymic mice inoculated with cells from a human ovarian cancer cell line. Our results also indicate that every second-day therapy, and, therefore lower total dose, of LY294002, prevents dematological side effects. Thus, combining a PI3k inhibitor with an anticancer chemotherapeutic agent may be an effective way to control tumor growth as well as ascites formation in ovarian cancer patients with acceptable side effects.

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