Inhibition of Phosphatidylinositol 3-Kinase-Akt Signaling Blocks Growth, Promotes Apoptosis, and Enhances Sensitivity of Small Cell Lung Cancer Cells to Chemotherapy

Geoffrey W. Krystal, Geoffrey Sulanke, and Julie Litz

Department of Medicine, Medical College of Virginia/Virginia Commonwealth University [G. W. K., G. S.], and McGuire Veterans Affairs Medical Center [G. W. K., J. L.], Richmond, Virginia 23249

Abstract
A promising therapeutic alternative to inhibition of growth factor receptors is the inhibition of downstream signal transduction pathways. Such an approach may be especially important in tumors that can use signals from multiple growth factor receptors for growth and survival. Both stem cell factor (SCF) and insulin-like growth factor (IGF)-I, components of prominent small cell lung cancer (SCLC) autocrine loops, as well as FCS, can potently activate phosphatidylinositol 3-kinase (PI3K)-Akt signaling, albeit with different kinetics. SCF-induced PI3K-Akt activation occurs rapidly but fades within 60 min; IGF-I and FCS-induced activation persists for at least 6 h. SCF and IGF-I-mediated growth was potently inhibited by LY294002 in proportion to its ability to inhibit phosphatidylinositol 3-kinase (PI3K)-Akt signaling. A panel of six SCLC cell lines grown in 10% FCS was also very sensitive to PI3K-Akt signaling, albeit with different sensitivities. SCF-induced PI3K-Akt activation occurs rapidly but fades within 60 min; IGF-I and FCS-induced activation persists for at least 6 h. SCF and IGF-I-mediated growth was potently inhibited by LY294002 in proportion to its ability to inhibit phosphatidylinositol 3-kinase (PI3K)-Akt signaling. A panel of six SCLC cell lines grown in 10% FCS was also very sensitive to LY294002, with average IC50 and LD50 of 5 and 25 μM, respectively. These drug concentrations suppressed the growth of the MRC-5 pulmonary fibroblast cell line and primary bronchial epithelial cells but did not induce significant cell death. Because LY294002 can also inhibit PI3K-related enzymes, we confirmed the role of the PI3K-Akt pathway in SCLC using doxycycline-regulated expression of a dominant-negative (kinase dead) and a constitutively active (CA; myristilated) Akt allele. Expression of dominant-negative Akt, which could only be achieved at relatively low levels, completely inhibited growth in the absence of exogenous growth factors and inhibited SCF-mediated growth but had no effect on IGF-I-mediated growth at the expression levels attained. Expression of CA Akt markedly augmented growth in the absence of exogenous growth factors but had minimal effect on growth in the presence of saturating concentrations SCF or IGF-I. Because PI3K-Akt signaling is known to promote survival under apoptotic stresses, we determined the effect of this pathway on SCLC sensitivity to etoposide. LY294002 potentiated the effect of low concentrations of etoposide in inhibiting growth and inducing apoptosis. The effect of low concentrations of LY294002 could largely be reversed by expression of CA Akt, suggesting that it was mediated by inhibition of Akt signaling. Expression of CA Akt by itself also induced resistance to etoposide-mediated apoptosis. Taken together, these data demonstrate that PI3K-Akt signaling promotes SCLC growth, survival, and chemotherapy resistance. Therefore, selective inhibitors of PI3K or Akt could potentially be useful as novel therapeutic agents in the treatment of SCLC.

Introduction
SCLC accounts for approximately 20–25% of all lung cancers and, despite high initial response rates to chemotherapy, causes the demise of 90–95% of affected individuals (1). Among the causes for its aggressive clinical course are likely to be the loss of both Rb and p53 tumor suppressor function, overexpression of Bcl-2 and Myc family genes, and the existence of multiple autocrine loops (2). These genetic aberrations result in a lack of response to negative growth regulatory signals and the continuous presence of positive signals that regulate growth, motility, and invasion. One of the more common and better characterized autocrine loops consists of coexpression of SCF and its cognate receptor tyrosine kinase, Kit. Several studies have demonstrated that activation of Kit can lead to enhanced proliferation, inhibition of apoptosis, and enhanced motility of SCLC cells (3–6). These observations have led to the identification of potent selective small molecule inhibitors of Kit, which are effective inhibitors of SCLC growth in vitro and could play an important role in the treatment of this malignancy (7, 8). However, these studies have also demonstrated that other growth factors present in serum, including IGF-I, can partially com-

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2 To whom requests for reprints should be addressed, at Richmond Veterans Affairs Medical Center (111K), 1201 Broad Rock Boulevard, Richmond, VA 23249. Phone: (804) 675-5446; Fax: (804) 675-5447; E-mail: glkrystal@hscc.vcu.edu.

3 The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-SCLC; Rb, retinoblastoma; SCF, stem cell factor; PI3K, phosphatidylinositol 3-kinase; IGF, insulin-like growth factor; IP, immunoprecipitation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; DN, dominant negative; CA, constitutively active; PARP, poly(ADP-ribose) polymerase; GSK, glycogen synthase kinase; ATM, ataxia-telangiectasia mutated; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end labeling.
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SCLC. SCLC has an approximately 10-fold increase in proliferative and antiapoptotic signaling in a variety of signaling plays a critical role in Kit-mediated proliferation, survival, and differentiation in murine family member Bad (16). Mutation of the Kit phosphotyrosine binding site for the p85 regulatory subunit of PI3K, which mediates its antiapoptotic effects in c-kit-transfected HEK 293 cells by phosphorylation of the proapoptotic Bcl-2 family member Bad (16). Mutation of the Kit phosphotyrosine binding site for the p85 regulatory subunit of PI3K, murine Tyr719, and human Tyr721 (17) results in diminished SCF-mediated proliferation of mast cells and a markedly decreased resistance to apoptotic stimuli (18). Knockout of the p85 subunit results in a similar mast cell phenotype (19). In addition, mice homozygous for the Kit Y719F mutation have severe defects in gametogenesis (20), confirming that PI3K inhibition was consistent with the observation that IGF-I, which is a potent activator of PI3K in SCLC (10). However, IGF-I is known to be a potent activator of PI3K and the downstream kinase Akt, the activation of which is a critical component of antiapoptotic signaling in fibroblasts (12). Kit is also known to be a potent activator of PI3K (13–15) and Akt, which mediates its antiapoptotic effects in c-kit-transfected HEK 293 cells by phosphorylation of the proapoptotic Bcl-2 family member Bad (16). Mutation of the Kit phosphotyrosine binding site for the p85 regulatory subunit of PI3K, murine Tyr719, and human Tyr721 (17) results in diminished SCF-mediated proliferation of mast cells and a markedly decreased resistance to apoptotic stimuli (18). Knockout of the p85 subunit results in a similar mast cell phenotype (19). Evidence also suggests that the PI3K-Akt pathway may be important in proliferative and antiapoptotic signaling in SCLC. SCLC has an approximately 10–15% incidence of mutation of the PTEN phosphatase, which negative regulates the PI3K pathway (21, 22). Extracellular matrix proteins, which can activate PI3K and Akt through integrin-mediated activation of Fak (23), were shown to confer resistance to chemotherapy-induced apoptosis on SCLC cell lines (24). A screening study demonstrated that SCLC cell lines appear to have elevated levels of PI3K activity, and inhibition of PI3K activity with the pharmacological inhibitors wortmannin and LY294002 inhibited growth and induced apoptosis of cells grown in serum-free medium supplemented with insulin and transferrin (25). The reasons for the constitutive activation of PI3K in this study were never addressed but could be related to the high concentration of insulin in the culture medium. Thus, it is still unclear whether SCLC does display constitutive activation of PI3K. A reasonable hypothesis, however, is that autocrine growth loops, such as those consisting of coexpression of SCF or IGF-I and their cognate receptors, provide the stimulus for PI3K activation. In addition, the role of the PI3K-Akt pathway in proliferative and antiapoptotic signaling in SCLC is not entirely clear because pharmacological inhibitors of PI3K also inhibit related enzymes such as the DNA-dependent protein kinase and ATM (26, 27), which could play a role in these processes. For these reasons, we have explored the role of SCF and IGF-I–mediated PI3K-Akt activation in proliferative and antiapoptotic signaling in SCLC by using conditionally expressed DN and CA Akt mutants in addition to biochemical inhibitors.

Materials and Methods

Cell Growth. SCLC cell lines were grown in RPMI 1640 supplemented with 2 mM l-glutamine, with (complete medium) or without 10% fetal bovine serum (HyClone, Logan, Utah); when grown in the absence of serum, 0.1% BSA (Sigma Chemical Co., St. Louis, MO) was added to the medium. Where indicated, serum-free medium was supplemented with recombinant SCF (Peprotech, Rocky Hill, NJ) or IGF-I (R&D, Minneapolis, MN) at the indicated concentrations. The H146, H187, H209, H510, and H526 cell lines have been characterized previously (28). The WBA SCLC cell line was derived from the involved bone marrow aspirate of an untreated patient (29). Primary normal human bronchial epithelial cells and bronchial epithelial medium were purchased from BioWhittaker (Walkersville, MD). MRC-5 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Eagle’s minimal essential medium with 2 mM l-glutamine, Earle’s balanced salt solution, and 10% fetal bovine serum. Cells were stimulated with growth factors after preincubation in serum-free medium overnight. Cell growth was measured using the MTT (Sigma) colorimetric dye reduction method, an assay shown to correlate very well with viable SCLC cell number under the conditions used (30). Duplicate plates containing eight replicate wells/assay condition were seeded at a density of 1 × 10^6 cells in 0.1 ml of medium, and data were expressed as the change in absorbance at 540 nm over 72 h, relative to initial values obtained 3 h after plating. LY294002, wortmannin, and etoposide (all from Calbiochem, San Diego, CA) were solubilized in DMSO; the final concentration of DMSO in all cultures, including controls, was 0.1%.

Clonogenic Assay. H526 cells (1 × 10^5) were treated with DMSO vehicle (control), 1 μM LY294002, 0.3 μM etoposide, or a combination of LY294002 and etoposide for 48 h in complete medium. The cells were then washed and resuspended in 3 ml of 0.25% agarose in complete medium and plated over a layer of 0.5% agarose in medium in 60-mm plates. Plates were incubated for 14 days, and viable (blue) macroscopic colonies were counted 2 h after overlay with MTT dye. Assays were performed in triplicate.

TUNEL Assay. Terminal deoxynucleotidyl transferase-mediated labeling of free DNA 3’ ends with fluorescein-conjugated dUTP was accomplished using the In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany). Cytospin preparations were fixed and labeled according to the manufacturer’s directions. Four independent ×100 fields containing a minimum of 300 cells on each of two replicate slides were evaluated for nuclear labeling by fluorescence microscopy for each treatment or condition.

Generation of Cell Lines Expressing Mutant Akt. DN (K179M) and CA (containing the src myristolation signal) murine Akt1 cDNAs cloned into the pUSE mammalian expression vector and containing a Myc-tag were purchased.
from UBI (Lake Placid, NY). For doxycycline-regulated expression, the previously described H526-IC4 subclone (10) constitutively expressing the reverse Tet repressor was used. The DN and CA Akt1 cDNAs were excised from the pUSE vector and cloned into the pTRE (Clontech, Palo Alto, CA) expression vector. The resulting expression vectors were then electroporated into H526-IC4 cells along with the pSV2-Hygro hygromycin resistance plasmid using conditions described previously (10). Hygromycin-resistant subclones were selected by limiting dilution in 600 µg/ml hygromycin (Calbiochem) and screened for doxycycline-inducible expression of Myc-tagged Akt by Western blotting. Induction of mutant Akt expression was accomplished by overnight incubation in 2 µg/ml doxycycline (Sigma). In all experiments studying the effects of mutant Akt expression, doxycycline was present continuously.

**Immunoprecipitation and Western Blotting.** Cells were lysed for IP in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP40, 1.5 mM MgCl₂, 0.1% SDS, 0.08 M Tris-HCl (pH 6.8), 0.1 M DTT, and 10% glycerol, 0.2 mM NaVO₄, 100 µM/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 10 µg/ml leupeptin using a Dounce homogenizer with a tight-fitting pestle; protein concentrations were determined by BCA assay (Pierce, Rockford, IL). The lysate, containing 1–1.5 mg of protein, was centrifuged for 10 min at 10,000 × g to obtain a soluble postnuclear supernatant. IP was initiated by addition of 10 µg of monoclonal anti-Kit antibody (K45; NeoMarkers, Frement, CA), followed by incubation for 2 h at 4°C and on an additional 2 h in the presence of protein A–G agarose. The IP was washed four times in lysis buffer and then once in SDS sample loading buffer [2% SDS, 0.08 µl Tris-HCl (pH 6.8), 0.1% DTT, and 10% glycerol] was added; the IP was then resolved on a 10% polyacrylamide gel. For preparation of whole cell lysates, cells were resuspended in ice-cold PBS and an equal volume of 2× SDS sample loading buffer was added, followed by shaking through a 25-gauge needle. Western blotting was performed using standard procedures, with detection using the ECL chemiluminescent system (Amersham, Arlington Heights, IL) and visualization using a Fuji cooled CCD camera and the Aida 2.0 software package (Raytest, Inc., New Castle, DE). Staining was accomplished using the following antibodies: anti-p85 PI3K regulatory subunit and anti-Myc-tag, polyclonals (UBI); anti-Kit, 3D6 monoclonal (Boehringer Mannheim, Indianapolis, IN); anti-actin, monoclonal (Sigma); anti-pan GSK, polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-Akt Thr-308, Ser-473, pan-Akt, anti-phospho GSK α/β, anti-cleaved PARP, caspase 3, cleaved caspase 3, polyclonals (Cell Signaling, Beverly, MA).

**Results**

**SCF and IGF-I Activate Akt via PI3K in H526 Cells.** Activation of Kit by SCF results in a rapid recruitment of the p85 regulatory subunit of PI3K to the receptor that is dependent on the phosphorylation of Tyr-721 in c-kit-transfected COS cells (17). To initially determine whether SCF activates PI3K in SCLC, we used the H526 SCLC cell line, which expresses high levels of Kit and can grow in medium containing SCF as the sole growth factor. Cells made quiescent in growth fac-

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**Fig. 1.** SCF and IGF-I activate Akt via PI3K in H526 cells. A, Western blot of a Kit immunoprecipitation before and after SCF stimulation stained for p85 and Kit, illustrating that p85 (α and β) associates with Kit only after SCF stimulation. B, cell lysates stained for active (pThr308 and pSer473) and total Akt and phosphorylated and total GSK before and after SCF or IGF-I stimulation in the absence or presence of 100 nM wortmannin. C, cell lysates stained for active (pSer473) and total Akt and phosphorylated and total GSK before and after SCF or IGF-I stimulation in the absence or presence of the indicated concentrations of LY294002.

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**The Time Course of Akt Activation in SCLC Is Growth Factor Dependent.** In preliminary experiments, we noted variable results when the ability of SCF, IGF-I, and FCS to activate Akt was compared (Ref. 7 and data not shown). We hypothesized that this variability could be attributable to a different time course of activation. Fig. 2A illustrates one of several time course experiments performed. Typically, SCF-induced Akt activation peaked at 5–10 min, began fading between 15 and 30 min, and returned to baseline by 60 min after stimulation. Kinetics of IGF-I-mediated activation were
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and cell death, with an average IC50 of that all cell lines showed dose-dependent growth inhibition to increasing concentrations of LY294002. Fig. 3 illustrated growth of a broad sampling of SCLC cells, we exposed whether the PI3K inhibitor could also inhibit serum-stimulated growth. To determine whether PI3K was involved in SCLC growth, we initially exposed H526 cells to stimulated SCLC Growth.

LY294002 Efficiently Inhibits SCF, IGF-I, and Serum-stimulated SCLC Growth. To determine whether PI3K was involved in SCLC growth, we initially exposed H526 cells to SCF or IGF-I and increasing concentrations of LY294002 in serum-free medium and measured Akt activation by Western blot and growth using a 72-h MTT assay. As illustrated in Fig. 3A, LY294002 efficiently inhibited growth in a dose-dependent manner that correlated very well with inhibition of Akt phosphorylation. IGF-I-mediated Akt activation and growth were more resistant to LY294002, but 25 μM LY294002 was lethal to both SCF and IGF-I-treated cells. To determine whether the PI3K inhibitor could also inhibit serum-stimulated growth of a broad sampling of SCLC cells, we exposed six cell lines derived from both chemotherapy-treated and cycled growth of a broad sampling of SCLC cells, we exposed whether the PI3K inhibitor could also inhibit serum-stimulated growth. To determine whether PI3K was involved in SCLC growth, we initially exposed H526 cells to stimulated SCLC Growth.

epithelial cells were somewhat more sensitive to the growth-suppressive effects of LY294002. Yet, they did not show significant apoptosis until 50 μM LY294002 was reached, similar to the behavior of the most resistant SCLC cell line (H146). These results suggest that SCLC growth and survival mediated by a broad range of growth factors are selectively sensitive to inhibition of PI3K. However, because LY294002 inhibits related enzymes including the DNA-dependent protein kinase and the ATM kinase, we sought to confirm these results using an alternative strategy to inhibit the PI3K-Akt pathway.

Expression of a Constitutively Active Akt Allele Promotes, and a Dominant-Negative Allele Inhibits, SCLC Growth. Our initial strategy was to interfere with PI3K signaling by expression of a DN allele of the p110 catalytic subunit. However, despite several transfections of H526 cells using constitutive and conditional expression vectors, we failed to obtain clones with stable expression of the DN protein. We therefore moved downstream in the pathway,
using not only a DN Akt allele but also a CA allele to modulate pathway signaling. We again failed to isolate stable clones expressing the DN allele driven by the constitutive cytomegalovirus promoter, but we were able to isolate several clones expressing the CA allele, which grew markedly better in the absence of growth factors than the vector control (data not shown).

One possible explanation for the inability to isolate clones expressing DN p110 or Akt is that inhibition of the pathway during selection prevented the outgrowth of stable transfec-
tants. To avoid this problem, we cloned the DN and CA Akt alleles into the pTRE vector that allows doxycycline-induced expression in H526 cells expressing the TET-ON transcriptional regulator (10). Transfectants were selected in the absence of doxycycline and several clones capable of doxycycline-regulated expression of both alleles were isolated. Fig. 4 illustrates the levels of doxycycline-induced expres-
sion in two transfectants for each allele relative to the vector control and relative to endogenous levels of Akt. The trans-
ected alleles contain an epitope tag and therefore have a slower electrophoretic mobility, allowing the heterologous protein to be distinguished from the endogenous protein after staining for total Akt. Staining for the Myc-epitope tag confirmed the slower mobility form as the heterologous pro-
tein, and staining for phospho-Akt confirmed that the CA protein was constitutively phosphorylated (not shown). As illustrated in Fig. 4A, high levels of the CA Akt could be expressed relative to the endogenous protein, whereas only clones capable of a relatively low level expression of DN Akt were isolated. To document that expression of the trans-
ected alleles had the predicted effect on the PI3K-Akt path-
way, we studied the phosphorylation of the Akt target GSK3 (Fig. 4B). Expression of CA Akt induced phosphorylation of GSK3β in the absence of growth factors and augmented both SCF and IGF-I-mediated GSK3β phosphorylation. In-
duced expression of DN Akt significantly reduced GSK3β phosphorylation in response to SCF but only had a minor effect on GSK3 phosphorylation induced by IGF-I. The in-
ability to significantly alter IGF-I-mediated GSK3 phosphory-
lation may be attributable to the potent activation of the PI3K-Akt pathway by IGF-I and the relatively low levels of expression of the DN protein.

Growth of the Akt transfectants and the vector control was compared in the absence of growth factors and in the presence of SCF or IGF-I (Fig. 5). In the absence of doxycycline and growth factors, the clones containing the active allele showed a moderate growth enhancement, and the clones expressing the DN protein showed modest growth suppres-
sion. This is likely to be attributable to low level expression of the heterologous proteins in the uninduced state (Fig. 4A). In the presence of doxycycline (in the absence of growth fac-
tors), these effects were magnified. In the presence of SCF, the expression of CA Akt minimally enhanced growth and in the presence of IGF-I did not enhance growth. These data demonstrate that enhancement of Akt activation beyond that...
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Inhibition of PI3K-Akt signaling enhances etoposide-mediated cytotoxicity. A, clone CA 1A1 cells in complete medium containing vehicle or 1 μM LY294002 were incubated in the absence or presence of doxycycline and increasing concentrations of etoposide. Change in relative viable cell number was assessed by a 72-h MTT assay. B, clone CA 1B4 cells in complete medium containing vehicle, 1, or 10 μM LY294002 were incubated in the absence or presence of doxycycline and increasing concentrations of etoposide. Change in relative viable cell number was assessed by a 72-h MTT assay. Columns, means; bars, SE.

produced by these growth factors had little influence on growth, at least at the levels of expression attainable in the inducible system. Expression of DN Akt did have a significant repressive effect on growth in SCF, consistent with its suppression of GSK3 phosphorylation (Fig. 4B). Expression of DN Akt had no effect on growth in IGF-1, consistent with its failure to significantly repress IGF-I-mediated GSK3 phosphorylation.

**Inhibition of PI3K Enhances Basal and Etoposide-mediated Apoptosis.** It is now understood that one of the primary activities of chemotherapy is to selectively induce tumor cell apoptosis (32). Evidence also suggests that the activation of the PI3K-Akt pathway in several tumor types induces resistance to apoptosis initiated by chemotherapeutic agents. To address the role of Akt in determining SCLC sensitivity to apoptosis, we initially exposed the two clones expressing the CA Akt allele to various concentrations of LY294002 and etoposide in the absence and presence of doxycycline (Fig. 6). Both inducible clones behaved similarly except that growth of the 1A1 clone in serum-containing medium could be further stimulated by induction of active Akt (Fig. 6A), whereas the growth of the 1B4 clone could not be further stimulated in serum-containing medium (Fig. 6B).

Addition of 10 μM LY294002 markedly suppressed growth (Fig. 6B) and induced the appearance of morphologically apoptotic cells (not shown). However, in both clones, addition of 1 μM LY294002 had little or no effect on growth, but coincubation with 1 μM LY294002 and etoposide resulted in enhanced cytotoxicity relative to vehicle controls, especially at low etoposide concentrations. Induction of CA Akt expression not only substantially reversed this enhancement of cytotoxicity by LY294002 but also clearly protected against etoposide-mediated cytotoxicity of vehicle controls at all etoposide concentrations. This is especially evident in the 1B4 clone, where induction of CA Akt produced no growth enhancement in serum-containing medium. Taken together, these observations demonstrate that activation of Akt protects SCLC cells from etoposide-mediated cytotoxicity and that inhibition of PI3K-Akt signaling markedly enhances etoposide-mediated cytotoxicity. Furthermore, because the enhancing effects of 1 μM LY294002 can be largely reversed by expression of CA Akt, it appears that at low concentrations of LY294002, potentiation of etoposide-mediated cytotoxicity is almost entirely attributable to inhibition of the PI3K-Akt pathway.

To determine whether the combination of LY294002 and etoposide resulted in enhanced apoptosis, we performed a TUNEL assay on CA Akt and control clones incubated for 24 h in the presence of 10 μM etoposide, 1 μM LY294002, or the combination. As illustrated in Fig. 7A, addition of LY294002 to etoposide resulted in enhanced apoptosis relative to etoposide alone. Doxycycline-induced expression of CA Akt reduced apoptosis by ~50% in all cultures, including the basal rate of apoptosis seen in the vehicle controls. To confirm the results of the TUNEL assay, we determined the level of caspase-3 activation and PARP cleavage induced by the combination of LY294002 and etoposide (Fig. 7B). Addition of 1 μM LY294002 clearly enhanced etoposide-mediated caspase-3 and PARP cleavage. This enhancement was largely reversed by doxycycline-induced expression of CA Akt. These results are consistent with those of the MTT and TUNEL assays, suggesting that a low concentration of LY294002 can potentiate the apoptotic effect of etoposide by specific inhibition of PI3K-Akt signaling. To confirm this observation in cells not genetically manipulated, parental H526 cells were exposed to DMSO (vehicle control), 1 μM LY294002, 0.3 μM etoposide, or the combination for 48 h in complete medium and then plated in soft agarose for a 14-day clonogenic assay (Table 1). One μM LY294002 had no effect on clonogenic growth but decreased colony number by >80% when combined with a low concentration of etoposide, relative to etoposide treatment alone. Taken together, these data strongly indicate that a low concentration of LY294002, insufficient to induce growth inhibition, can markedly enhance the growth-suppressive and apoptotic effects of etoposide.

**Discussion**

Signaling pathways activated by growth factor receptors play an important role in regulating tumor cell growth, sur-
and cleaved PARP by Western blotting.

Indicated. Cell lysates were probed for both cleaved (active) caspase-3

10

the CA 1A1 clone was incubated for 24 h in complete medium containing

Columns

apoptosis was assessed by TUNEL assay.

- SCLC growth, produced a delayed but also a prolonged

- Activation of the signaling pathway. The reason for the de-

- Prolonged presence of the growth factor, there was also rapid

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- Activates the PI3K-Akt pathway, which can be activated by SCF, IGF-I, and

- Constitutive expression of the CA Akt allele

- Concluded that the PI3K-Akt pathway was required for the clonal

- Each tissue culture was inoculated at 25,000 cells per well for 7 days,

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- Administration of the PI3K inhibitor LY294002 was also more resistant to inhibition by LY294002, with IGF-I-mediated activation being more resistant to the drug (Fig. 3A).

- SCLC cell lines grown in serum-containing medium were uniformly extremely sensitive to LY294002, with average IC_{50} and LD_{50} values of 5 and

- LY294002 enhances the efficacy of etoposide in clonogenic assays

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle control</th>
<th>1 μM LY294002</th>
<th>0.3 μM LY294002</th>
<th>LY294002 and etoposide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony number ± SD</td>
<td>289 ± 28</td>
<td>303 ± 37</td>
<td>97 ± 13</td>
<td>18 ± 7</td>
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- Although we have not yet established a direct link between the kinetics of PI3K-Akt activation and potency as a growth factor nor demonstrated the reasons for the different kinetics, evidence gathered in multiple biological systems suggests that the kinetics of the signal transduction pathway activation is a critical determinant of biological response (33).

- We have assessed the importance of the PI3K pathway using two complementary approaches. The first and simplest approach was the use of the PI3K inhibitor LY294002. Significantly, in serum-free medium supplemented with SCF or IGF-I, the degree of growth inhibition correlated very well with the degree of inhibition of PI3K-Akt activation induced by LY294002, with IGF-I-mediated activation being more resistant to LY294002, little or no cytotoxicity was induced. In contrast, 50 μM LY294002 did induce some cytotoxicity in primary bronchial epithelial cells, but this same drug concentration produced almost universal cell death in SCLC cell lines (Fig. 3B).

- The difficulty in attributing this cell death entirely to inhibition of PI3K signaling is that LY294002 can inhibit related enzymes, such as the DNA-dependent protein kinase and the ATM kinase (26, 27), making it impossible to definitively determine the pathway responsible for the cytotoxic response at higher drug concentrations. To do so, we attempted to express DN p110 and Akt alleles under the regulation of a constitutive promoter without success, suggesting that the PI3K-Akt pathway was required for the clonal growth of SCLC. Constitutive expression of the CA Akt allele resulted in enhanced growth in the absence of exogenous growth factors, emphasizing the importance of the pathway.

- However, the most informative approach was to express both DN and CA Akt under the regulation of a doxycycline-inducible promoter. Under these circumstances, assessment of the relative levels of endogenous GSK3-β phosphorylation

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**Fig. 7.** Expression of active Akt protects against enhancement of eto-

- Poside-mediated apoptosis by LY294002. A, clone CA 1A1 or vector
cell lines were incubated for 24 h in complete medium containing
doxycycline, 10 μM etoposide, and 1 μM LY294002 as indicated, and

- TUNEL assay. Columns; bars, SE. B, the CA 1A1 clone was incubated for 24 h in complete medium containing

- Etoposide (Etop), 1 μM LY294002 (LY), and doxycycline (Dox) as

- Cell lysates were probed for both cleaved (active) caspase-3 and cleaved PARP by Western blotting.

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- Growth and signaling if added to serum-free medium and suggests that modifying factors in serum such as IGF-I binding

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cytotoxicity was noted at concentrations of 10 μM or greater, and this reflected the most selective action of LY294002. Although the growth of the MRC-5 pulmonary fibroblast cell line could be inhibited by as much as 70% at 50 μM LY294002, little or no cytotoxicity was induced. In contrast, 50 μM LY294002 did induce some cytotoxicity in primary bronchial epithelial cells, but this same drug concentration produced almost universal cell death in SCLC cell lines (Fig. 3B). The difficulty in attributing this cell death entirely to inhibition of PI3K signaling is that LY294002 can inhibit related enzymes, such as the DNA-dependent protein kinase and the ATM kinase (26, 27), making it impossible to definitively determine the pathway responsible for the cytotoxic response at higher drug concentrations. To do so, we attempted to express DN p110 and Akt alleles under the regulation of a constitutive promoter without success, suggesting that the PI3K-Akt pathway was required for the clonal growth of SCLC. Constitutive expression of the CA Akt allele resulted in enhanced growth in the absence of exogenous growth factors, emphasizing the importance of the pathway.

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could be used to monitor the degree of pathway activation or inhibition. Doxycycline-induced expression of CA Akt enhanced levels of GSK3-β phosphorylation and stimulated growth in the absence of growth factors. But there appeared to be a threshold effect because further stimulation of phosphorylation upon addition of SCF or IGF-I, beyond levels produced by the growth factors themselves, did not result in significant growth enhancement (Figs. 4 and 5). Expression of DN Akt inhibited SCF-mediated GSK-β phosphorylation and growth but had little effect on IGF-I-mediated GSK3-β phosphorylation and no effect on growth, presumably because of insufficient levels of DN expression. Taken together, these data demonstrate that a threshold degree of PI3K-Akt pathway activation must be attained for SCLC proliferation.

The inducible expression of the CA Akt allele was particularly helpful for distinguishing the proliferative from the antiapoptotic effects of the PI3K-Akt pathway. Much evidence has implicated PI3K-mediated Akt activation in protecting nonneoplastic and neoplastic cells from varied apoptotic stresses (35). Because of the therapeutic implications, we specifically explored the role of the PI3K-Akt pathway in modulating etoposide-mediated apoptosis. Expression of CA Akt partially protected cells from etoposide-mediated growth inhibition and apoptotic cell death (Figs. 6 and 7). Exposure of H526 cells to LY294002 enhanced etoposide-mediated growth suppression and apoptosis, especially at low (0.1–1 μM) concentrations of etoposide. Induction of CA Akt reversed the potentiating effect of 1 μM LY294002, indicating that the effect of this concentration of LY294002 was mediated largely by inhibiting Akt activation. Only partial inhibition of apoptosis was observed at 10 μM LY294002 (Fig. 6B), indicating that the higher drug concentration either more effectively blocked PI3K-mediated activation of antiapoptotic pathways not involving Akt or the drug was acting on PI3K-related enzymes. The latter explanation seems more plausible because etoposide induces apoptosis by inhibiting topoisomerase II (36), resulting in DNA double-strand breaks, and both the DNA-dependent protein kinase and the ATM kinase have been implicated in the recognition and repair of DNA strand breaks (37).

The above observations underscore the utility of combining expression of a CA Akt allele with LY294002 to confirm the specificity of the drug’s effects. Although numerous studies have used expression of DN PI3K and Akt alleles to confirm the specificity of biochemical PI3K inhibitors (35), it has been frequently difficult to completely reproduce the drug effects with the DN alleles. This has generally been attributed to insufficient levels of stable DN expression, a phenomenon we have encountered in this study not only using constitutive promoters but also regulated promoters to drive DN expression. Combining the readily induced high level expression of CA Akt with LY294002 allowed us to implicate inhibition of non-Akt signaling pathways in the effects of high concentrations of LY294002, especially with regard to apoptotic synergy with etoposide. This approach, however, does not allow a distinction between inhibition of PI3K-related enzymes versus PI3K-activated signaling pathways that parallel the Akt pathway.

In addition to demonstrating the importance of the PI3K-Akt pathway for proliferation and survival, our data suggest that Akt activation mediates resistance to etoposide, the most commonly used agent for the treatment of SCLC. We demonstrated that LY294002 not only enhanced the cytotoxicity of etoposide, but that induction of CA Akt inhibited etoposide-induced growth suppression and apoptosis. Recently, similar findings have been demonstrated in other tumor types using a variety of chemotherapeutic agents and strategies to inhibit PI3K-Akt signaling. Brognard et al. (34) demonstrated that a large majority of NSCLC cell lines tested displayed high constitutive levels of Akt activation, and LY294002 sensitized all cell lines tested to a variety of chemotherapeutic agents as well as ionizing radiation. They also used transient expression of a DN Akt allele to demonstrate enhancement of chemosensitivity in cell lines displaying high constitutive levels of Akt activation. Interestingly, cell lines without constitutive Akt phosphorylation were not sensitized by DN Akt, despite the observation that they were sensitized by high (≥10 μM) concentrations of LY294002. This observation suggests that LY294002 sensitizes NSCLC cells to chemotherapy based on its ability to inhibit both Akt activation and other signaling pathways, such as those initiated by the PI3K-related kinases, and is consistent with our data. This same study, as well as a second study (38), also demonstrated that expression of CA Akt induced chemotherapeutic resistance in NSCLC cell lines. Several studies have looked at the effects of PI3K-Akt inhibition on the chemosensitivity of ovarian cancer cell lines, in part because of the amplification of the AKT2 and mutations of the PTEN genes in this cancer. Two studies have demonstrated that expression of either CA Akt enhanced chemoresistance to etoposide (39) or DN Akt enhanced chemosensitivity (40) of ovarian cancer cell lines. However, a third study did not find any change in susceptibility to cisplatin or paclitaxel in the presence of LY294002 (41). A recent study has also demonstrated that LY294002 can enhance the efficacy of paclitaxel in a murine xenograft model of ovarian cancer (42). Two studies (43, 44) have demonstrated that LY294002 enhances the chemosensitivity of pancreatic and myeloid leukemia cell lines, respectively. Taken together, the current study and others indicate that activation of the PI3K-Akt pathway plays an important role in mediating chemotherapeutic resistance. However, it is also apparent that effects of PI3K inhibitors such as LY294002 on chemosensitivity cannot necessarily be attributed to inhibition of Akt activation, especially when used at concentrations of 10 μM or greater and combined with DNA-damaging agents that can activate DNA-dependent protein kinase and ATM. Uniquely, our data demonstrate that 1 μM LY294002, insufficient to induce growth inhibition or inhibit DNA-dependent protein kinase (26, 27), was sufficient to potentiate the activity of etoposide.

In summary, we have demonstrated that multiple growth factor receptors, including those activated by SCF, IGF-I, and FCS, can activate the PI3K-Akt pathway in SCLC, albeit with markedly different potencies. We have shown that a basal level of pathway activation is necessary for proliferation, and expression of a CA Akt allele is sufficient to drive proliferation of the H526 SCLC cell line. Using a novel com-

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bination of induced CA Akt expression and low concentrations of LY294002, we have demonstrated clearly that inhibition of Akt activation can enhance the apoptotic effects of chemotherapy. Furthermore, the low concentration of LY294002 used in these studies did not inhibit growth on its own and only partially inhibited Akt activation. Therefore, these data suggest that one way of improving the therapeutic index of PI3K inhibitors would be to combine them with other agents that induce apoptosis. On the basis of its critical role in proliferation, survival, and resistance to chemotherapy, it appears that the PI3K-Akt pathway would be an excellent target for novel drug development, which could have a profound effect on the treatment of SCLC and other malignancies.

References


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Geoffrey W. Krystal, Geoffrey Sulanke and Julie Litz