

# Akt/Protein Kinase B Is Constitutively Active in Non-Small Cell Lung Cancer Cells and Promotes Cellular Survival and Resistance to Chemotherapy and Radiation

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## ABSTRACT

To evaluate the role of Akt/PKB in non-small cell lung cancer (NSCLC) survival, we analyzed NSCLC cell lines that differed in tumor histology as well as *p53*, *Rb*, and *K-ras* status. Constitutive Akt/protein kinase B (PKB) activity was demonstrated in 16 of 17 cell lines by maintenance of S473 phosphorylation with serum deprivation. Additional analysis of five of these NSCLC lines revealed that phosphorylation of S473 and T308 correlated with *in vitro* kinase activity. Akt/PKB activation was phosphatidylinositol 3-kinase-dependent and promoted survival because the phosphatidylinositol 3 inhibitors LY294002 and wortmannin inhibited Akt/PKB phosphorylation, Akt/PKB activity, and increased apoptosis only in cells with active Akt/PKB. To test whether Akt/PKB activity promoted therapeutic resistance, LY294002 was added with individual chemotherapeutic agents or irradiation. LY294002 greatly potentiated chemotherapy-induced apoptosis in cells with high Akt/PKB levels, but did not significantly increase chemotherapy-induced apoptosis in cells with low Akt/PKB levels. Combined with radiation in cells with active Akt/PKB, LY294002 additively increased apoptosis and inhibited clonogenic growth. These results were extended with transiently transfected Akt/PKB mutants. Transfecting dominant negative Akt/PKB decreased Akt/PKB activity and increased basal apoptosis as well as chemotherapy- and irradiation-induced apoptosis only in cells with high Akt/PKB activity. Conversely, transfecting constitutively active Akt/PKB into cells with low Akt/PKB activity increased Akt/PKB activity and attenuated chemotherapy- and radiation-induced apoptosis. We therefore identify Akt/PKB as a constitutively active kinase that promotes survival of NSCLC cells and demonstrate that modulation of Akt/PKB activity by pharmacological or genetic approaches alters the cellular responsiveness to therapeutic modalities typically used to treat patients with NSCLC.

## INTRODUCTION

Lung cancer is the most lethal form of cancer for both men and women in the United States, with more than 150,000 deaths in 1998 (1). NSCLC<sup>2</sup> comprises the majority of lung cancer (over 75%), and when clinically extensive, it is typically characterized by inexorable disease progression despite treatment with chemotherapy and/or irradiation. Because chemotherapy and irradiation induce programmed cell death or apoptosis *in vitro* (2) and *in vivo* (3, 4), recent efforts have been made to understand whether molecular events in the apoptotic process are altered in NSCLC and whether these alterations might contribute to therapeutic resistance. NSCLC cell survival in the face of cytotoxic therapy is probably influenced by intrinsic properties

of the cell such as status of components of the apoptotic machinery, as well as extrinsic properties such as status of communication with the extracellular milieu mediated by extracellular matrix, GFs, and GFRs.

Alterations in tumor suppressor genes and cell cycle proteins have been identified in NSCLC specimens. Loss of function of tumor suppressor genes such as *p53* (5, 6), *K-ras* (7), *Fhit* (8), and *p16* (9), through mutation or through promoter silencing, and overexpression of cell cycle proteins such as cyclin D1 (10), cyclin E (11), and cyclin B1 (12), can decrease the cellular apoptotic potential and are common in NSCLC tumor specimens. The contribution of altered extracellular communication to apoptotic potential has not been well studied in NSCLC. The relative importance of various GFs and GFRs to NSCLC survival is less well understood, although expression of basic fibroblast GF and its receptor (13), epidermal GF-related GFs and erbB family members (14–16), platelet-derived GF (17, 18), vascular endothelial growth factor (14, 18), and transforming GF- $\beta$  (19) is increased in NSCLC tumor specimens. The role of IGF-I *in vivo*, which has been shown to confer resistance to chemotherapy and irradiation in other tumor systems (reviewed in Ref. 20), is complicated by regulation by multiple IGF-I-binding proteins. Nonetheless, IGF-I mRNA, IGF-II mRNA, and IGF-IR have been detected in a small number of NSCLC cell lines (21), and the relative ratio of IGF-I:IGF-I-binding protein 3 may be prognostic for the development of lung cancer when combined with other risks factors (22). The fact that multiple GFs may contribute to the development or progression of NSCLC highlight the importance of understanding how GF signals are transduced in NSCLC cells and in identifying which pathways are important for survival and contribute to therapeutic resistance. Because GFs stimulate multiple kinase cascades pathways, identification of kinases that are activated by different GFRs would identify points of convergence for survival pathways. These kinases would then make logical targets for approaches that attempt to abrogate kinase activity and increase apoptosis. Abrogating the activity of one kinase might then be effective in inhibiting survival signals originating from multiple signaling pathways. Among kinases identified to be downstream of GFR activation, Akt (or PKB) is the probably the best-characterized kinase known to promote cellular survival.

Akt/PKB is activated in response to activation by many different GFs, including IGF-I, epidermal GF, basic fibroblast GF, insulin, interleukin-3, interleukin-6, and macrophage-colony stimulating factor (reviewed in Ref. 23). Akt/PKB is the cellular homologue of the product of the *v-akt* oncogene (24–26) and has three isoforms: Akt1, -2, and -3 (or PKB $\alpha$ , - $\beta$ , and - $\gamma$ ). Akt2/PKB $\beta$  and Akt3/PKB $\gamma$  are overexpressed in ovarian, pancreatic, and breast cancer cells (27, 28). Activation of all three isoforms is similar in that phosphorylation of two sites, one in the activation domain and one in the COOH-terminal hydrophobic motif, are necessary for full activity. For Akt/PKB $\alpha$ , phosphorylation of T308 in the activation domain by PDK1 is dependent on the products of PI3-K, PIP<sub>2</sub> and PIP<sub>3</sub>. PIP<sub>2</sub> and PIP<sub>3</sub> bind to the pleckstrin homology domains of Akt/PKB and PDK1, which relieves steric hindrance, fully activates PDK1, and translocates Akt/PKB to the plasma membrane. The mechanism of S473 phosphorylation is less clear. Kinases potentially responsible for S473 phosphor-

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<sup>2</sup> The abbreviations used are: NSCLC, non-small cell lung cancer; PKB, protein kinase B; PI3-K, phosphatidylinositol 3-kinase; PIP<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate; PIP<sub>2</sub>, phosphatidylinositol 3,4-bisphosphate; PDK1, phosphoinositide-dependent kinase-1; HA, hemagglutinin; PKC, protein kinase C; GFP, green fluorescent protein; GF, growth factor; GFR, growth factor receptor; IGF-I, insulin-like growth factor-I; SHIP, SH2-containing inositol 5-phosphatase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PP2A, protein phosphatase 2A; CDDP, *cis*-platinum(II)-diamine dichloride; Myr, myristylated; FBS, fetal bovine serum; TBS, Tris-buffered saline. RT-PCR, reverse transcription-PCR

ylation include PDK1 (29), integrin-linked kinase, or an integrin-linked kinase-associated kinase (30, 31), Akt/PKB itself (32) or an as-yet uncharacterized PDK2. Akt/PKB activation may also be achieved through PI3-K independent means, either through phosphorylation of Akt/PKB by kinases such as PKA (33) or CAM-KK (34), or under conditions of cellular stress (28, 35, 36). Interestingly, activation of Akt/PKB by PKA or CAM-KK does not appear to require phosphorylation of S473. The relative importance of PI3-K independent and dependent means of Akt/PKB activation *in vivo* is unclear. Once activated, however, Akt/PKB exerts antiapoptotic effects through phosphorylation of substrates such as Bad (37, 38) or caspase 9 (39) that directly regulate the apoptotic machinery, or substrates such as the human telomerase reverse transcriptase subunit (Ref. 40), forkhead transcription family members (41, 42), or I $\kappa$ B kinases (43, 44) that indirectly inhibit apoptosis.

Previous studies have demonstrated that Akt/PKB plays an important role in survival when cells are exposed to different apoptotic stimuli such as GF withdrawal; UV irradiation; matrix detachment; cell cycle discordance; DNA damage; and administration of anti-Fas antibody, transforming GF- $\beta$ , glutamate, or bile acids (45–59). However, the role of Akt/PKB in tumor cell survival and resistance to cancer therapy has not been well studied in any tumor system.

In these studies, we demonstrate that Akt/PKB is activated in most NSCLC cell lines. This activation is maintained when cells are serum-starved, and NSCLC cells use Akt/PKB for survival because when PI3-K inhibitors are added or kinase-dead Akt/PKB transfected, NSCLC cells undergo apoptosis. Manipulating Akt/PKB activity alters sensitivity to chemotherapy and irradiation: transfecting constitutively active Akt/PKB into NSCLC cells that have low endogenous Akt/PKB activity increases resistance to chemotherapy and irradiation; alternatively, adding a PI3-K inhibitor or transfecting kinase-dead Akt/PKB into cells with high levels of Akt/PKB activity results in dramatic sensitization to these modalities. These data show that targeting a specific kinase that promotes survival such as Akt/PKB can change the apoptotic potential of NSCLC cells, resulting in greater efficacy of chemotherapy and irradiation *in vitro*.

## MATERIALS AND METHODS

**Materials.** Human recombinant IGF-I was purchased from R&D Systems (Minneapolis, MN). LY294002 was purchased from Alexis Biochemicals (San Diego, CA). Wortmannin and CDDP were purchased from Sigma Chemical Co. (St. Louis, MO). Etoposide and paclitaxel were from Calbiochem (La Jolla, CA). Trastuzumab (Herceptin) was from Genentech (San Francisco, CA) and gemcitabine was from Eli Lilly (Indianapolis, IN). Akt/PKB, p70 S6 kinase, GSK-3 $\alpha/\beta$  antibodies and the Akt kinase assay kit were from New England Biolabs (Beverly, MA). Antibodies against Akt1 and Akt2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Akt3 were purchased from Calbiochem. PTEN antibodies were from Oncogene Research Products (Cambridge MA) and HA-probe F7 antibody was from Santa Cruz Biotechnology, Inc. Protease inhibitor cocktail was obtained from Sigma Chemical Co., and protein assay materials were from Bio-Rad (Hercules, CA). All cell culture reagents were purchased from Life Technologies, Inc. (Rockville, MD). Protran pure nitrocellulose membranes were purchased from Schleicher & Schuell (Dassel, Germany). Plasmid pEGFP-F encoding membrane-bound GFP was purchased from Clontech (Palo Alto, CA). pSR $\alpha$ , K179M, and Myr Akt/PKB plasmids were a kind gift from Dr. P. Tschlis, Fox Chase Cancer Center.

**Cell Culture.** All NSCLC lines were provided by H. Oie or Dr. F. Kaye at the National Cancer Institute/Naval Medical Oncology (Bethesda, MD). All cell lines were maintained in 75 cm<sup>2</sup> flask in Dulbecco's modified eagle medium (DMEM) and supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were incubated in a 37°C and 7.0% CO<sub>2</sub> atmosphere incubator. The stock cultures were split on a weekly basis at a 1:5 or 1:10 ratio. For radiation experiments, cells were irradiated with

a 6 MV linear accelerator (Department of Radiation Oncology, National Naval Medical Center, Bethesda, MD).

**Transient Transfections.** Cells were plated at a concentration of  $2 \times 10^5$  cells/well of a six-well dish (~60% confluency) and transfected using Superfect transfection reagent according to the manufacturer's protocol (Quiagen, Valencia, CA). For gating purposes in flow cytometry, one well/plate was transfected with GFP only. For all other wells, plasmids encoding pSR $\alpha$ , K179M, or Myr Akt/PKB were cotransfected with the plasmid encoding GFP (dilution, 1:100). Only cells that expressed GFP were analyzed for cell cycle distribution via flow cytometry. Each experimental condition was analyzed in parallel for immunoblotting, kinase assays, and flow cytometry. Experiments were repeated at least three times.

**Pharmacological Treatments.** To study the effects of serum deprivation and IGF-I on Akt/PKB activation, NSCLC cells were plated at concentrations of  $2-4 \times 10^5$  cells/well in a 6- or 12-well plate in 10% DMEM, and incubated for 24 h. The media was changed to DMEM with 10% FBS or 0.1% FBS, and the cells were incubated overnight. In some samples, IGF-I (10 nM) was added for 10 min before lysing. To study the effects of PI3K inhibitors on Akt/PKB activity and phosphorylation, LY294002 (25  $\mu$ M) or wortmannin (1  $\mu$ M) was added 2 h before lysing the cells for immunoblotting. To study the effect of LY294002 on apoptosis, cells were incubated in 0.1% FBS in DMEM in the absence or presence of LY294002 (25  $\mu$ M) for 48 h. For combination experiments with LY294002 and chemotherapy, LY294002 was added simultaneously with each chemotherapeutic agent and both were incubated for 48 h before quantification of apoptosis. Chemotherapeutic agents were added at concentrations that had been shown previously to cause little apoptosis when used singly in NSCLC cells.<sup>3</sup> For experiments with transiently transfected cells, etoposide, CDDP, and paclitaxel were added 24 h before immunoblotting or flow cytometry analysis. Transiently transfected A549 cells were exposed to chemotherapy for 48 h. Apoptotic assays were done in triplicate and repeated at least three times.

**Immunoblotting.** Cell extracts were prepared by washing cells with PBS and adding 100  $\mu$ l of 2 $\times$  Laemmli sample buffer supplemented with 2  $\mu$ l protease inhibitor cocktail/well as described previously (60). Lysates were sonicated for 15 s with a Vibra Cell sonicator. The protein yield was quantified using the Bio-Rad Dc protein assay kit. Equivalent protein was loaded, and the lysates were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Equivalent loading was confirmed by staining membranes with fast green as described previously (61). The membranes were blocked for 1 h in blocking buffer (1 $\times$  TBS, 5% milk, and 0.20% Tween 20) and placed in primary antibody (1 $\times$  TBS, 5% milk, and 0.10% Tween 20; 1:1000 antibody) overnight at 4°C. Nitrocellulose membranes were washed three times in wash buffer (0.10% NP40, 0.10% Tween 20, and 1 $\times$  TBS). Primary antibody was detected using horseradish peroxidase-linked goat antimouse or goat antirabbit IgG antibodies and visualized with the enhanced chemiluminescent detection system (Amersham-Pharmacia Biotech, Buckinghamshire, England). Immunoblot experiments were performed at least three times.

**In Vitro Kinase Assays.** Akt/PKB kinase assays were performed using manufacturers recommendations (New England Biolabs), with modifications described below. Cells were plated at a concentration of  $2-4 \times 10^5$  and treated as described. Cells were washed once with ice-cold PBS, and 200  $\mu$ l of ice-cold lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium PP<sub>i</sub>, 1 mM  $\beta$ -glycerol phosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin] was added to the cells for 10 min. Lysates were cleared and allowed to immunoprecipitate for 2–3 h at 4°C with anti-Akt antibody. Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer. Kinase reaction was performed for 30 min at 30°C in kinase buffer supplemented with 200  $\mu$ M ATP and 1  $\mu$ g GSK-3 $\alpha/\beta$  fusion protein. Reaction was terminated with 3 $\times$  SDS buffer. The samples were heated at 100°C for 5 min and loaded into a 12% acrylamide gel. Experiments were repeated at least three times.

**Apoptosis Assays.** Floating cells were collected, and adherent cells were harvested by trypsinization and then centrifuged at 1000  $\times$  g for 5 min. Cells were fixed in ice-cold 70% methanol, added dropwise, and then incubated at –20°C for 30 min. Cells were centrifuged and incubated with propidium iodide (25  $\mu$ g/ml) supplemented with RNase A (30  $\mu$ g/ml) for 30 min at room

<sup>3</sup> J. Brognard and P. Dennis, unpublished observations.

temperature. Quantification of sub-2N DNA was determined by flow cytometry analysis using a Becton Dickinson FACSort and by manual gating using CellQuest software. Gating was performed on blinded samples.

**Clonogenic Assays.** H157 cells were aliquoted at  $4 \times 10^5$  cells/well into 12-well plates and incubated overnight. Cells were irradiated in the presence or absence of LY294002 (25  $\mu$ M) and incubated for 24 h. Cells were trypsinized, counted, and replated in triplicate in predetermined cell numbers to yield 50–200 colonies/well. Plates were incubated for 8 days to allow clonogenic growth. Colonies were washed with cold PBS and fixed in 10% acetic acid and 20% ethanol solution. Colonies were stained with a 4% crystal violet solution and counted with the Stratagene Eagle Eye. Assays were repeated at least three times.

**Soft Agar Assays.** H1155 cells were aliquoted into 12-well plates at a concentration of  $5 \times 10^5$  cells/well, irradiated in the presence or absence of LY294002 (25  $\mu$ M), and incubated for 24 h. Before harvesting the cells, bottom agar (1.6% agar:0.4% peptone) was mixed 1:1 with 2 $\times$  DMEM (20% FBS, 2% Pen/Strep, and 4 mM HEPES buffer). Three ml agar/media was poured into each 60-mm dish. Agar was allowed to solidify and equilibrate at 37°C in 5% CO<sub>2</sub>. Cells were trypsinized and counted on a hemocytometer using trypan blue exclusion to determine viable cell number. Cells were mixed first with 2 $\times$  DMEM and then with top agar (0.8% agar:0.2% peptone). Samples were plated in triplicate, with  $5 \times 10^4$  cells and 3.5 ml of cell mix added per plate. Plates were incubated 8–10 days to allow colony growth. Twenty-five squares (2 mm  $\times$  2 mm) were counted by eye per plate. Soft agar assays were performed three times.

**RT-PCR.** Total RNA was extracted from NSCLC cell lines subsequent to the Trizol Reagent (Life Technologies, Inc.) protocol. The quality and quantity of the RNA was determined by measuring the absorbance of the total RNA at 260 and 280 nm. For RT-PCR, the One-Step RT-PCR with the Platinum Taq kit (Life Technologies, Inc.) was used according to the manufacturer's instructions. Primers for Akt isoform expression were described by Okano *et al.* (62) and were synthesized by Sigma Chemical Co.-Genosys (TXF). The primers were: 5'-GCTGGACGATAGCTTGGGA-3' (Akt1 sense); 5'-GATGACAGAT-AGCTGGTG-3' (Akt1 antisense); 5'-GGCCCCTGATCAGACTCTA-3' (Akt2 sense); 5'-TCCTCAGTCGTGGAGGAGT-3' (Akt2 antisense); 5'-GCAAGTGGACGAGAATAAGTCTC-3' (Akt3 sense); and 5'-ACAATGTGGGCTCATGACTTCC-3' (Akt3 antisense).  $\beta$ -Actin primers were 5'-GTGGGGCGCCCCAGGCACCA-3' (sense) and 5'-CTCCTTAAGTCACG-CAGATTTC-3' (antisense). RT-PCR reactions contained 1  $\mu$ g of total RNA, 0.2  $\mu$ M of primer, 1  $\mu$ l of RT-Taq Mix, and 25  $\mu$ l of Reaction Mix. The cycling conditions for PCR were as follows: cDNA synthesis and predenaturation (1 cycle at 50°C for 30 min and at 94°C for two min); PCR amplification (40 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 45 s). The PCR products were electrophoresed on a 1% agarose gel

and visualized with ethidium bromide. The Akt primers were designed to generate 383- (Akt1), 276- (Akt2), and 329- (Akt3) bp products, respectively.

**Statistical Analysis.** Statistical comparison of mean values was performed using the Student *t* test. All *P*s are two-tailed. Interactions between LY294002 and chemotherapeutic agents were classified by the Fractional Inhibition Method as follows: when expressed as the fractional inhibition of cell viability, additive inhibition produced by LY294002 and chemotherapy (*i*) occurs when  $i_{1,2} = i_1 + i_2$ ; synergism occurs when  $i_{1,2} > i_1 + i_2$ ; and antagonism occurs when  $i_{1,2} < i_1 + i_2$  (63).

## RESULTS

**Akt/PKB Activity Is Constitutive and PI3-K Dependent in NSCLC Cells.** To determine whether Akt/PKB is active in NSCLC cells, we analyzed a panel of NSCLC cell lines established at the National Cancer Institute/National Naval Medical Center (64). Cell lines were chosen that varied in histology and in status of molecules commonly mutated in NSCLC (65). Cells were grown under normal growth conditions (10% FBS) or deprived of serum overnight (0.1% FBS), and Akt/PKB activity was assessed by immunoblotting with phospho-specific antibodies against phosphorylated S473. Table 1 shows that under normal growth conditions, 17 of 19 NSCLC cell lines (89%) exhibit phosphorylation of S473. Phosphorylation of S473 was maintained in 16 of 17 positive cell lines (94%) when cells were deprived of serum. Levels of native Akt/PKB varied slightly between cell lines, but there was no variation under these experimental conditions (data not shown). There was no apparent correlation of Akt/PKB phosphorylation with histological subtype or *p53*, *Rb*, or *K-ras* status. To ensure that the cell lines that did not exhibit S473 phosphorylation were capable of phosphorylating S473 upon stimulation, IGF-I was added, and S473 phosphorylation was measured. In all 10 cell lines tested, IGF-I increased S473 phosphorylation irrespective of endogenous levels, indicating that NSCLC cell lines phosphorylate Akt/PKB in response to IGF-I, and that the IGF-IR signaling pathway that leads to Akt/PKB activation is intact, even in cells that exhibit no Akt/PKB phosphorylation (H1355). The fact that S473 phosphorylation was maintained in 94% of NSCLC cell lines under serum deprivation, a condition where Akt phosphorylation is commonly decreased or absent, suggested that Akt/PKB was constitutively active in NSCLC cells, and that it might play a role in NSCLC survival.

Because full Akt/PKB activation requires phosphorylation of sites

Table 1 Survey of NSCLC cell lines for phosphorylation of Akt/PKB at S473

Lysates were prepared from cells exposed to 10% FBS (HS), 0.1% FBS overnight (LS), or LS + IGF-I (10 nM) for 10 min. Immunoblotting was performed with antibodies against phospho-S473 Akt/PKB and native Akt/PKB. + indicates phosphorylation of Akt/PKB at serine 473. – indicates that there was no detectable Akt/PKB phosphorylation at S473. NT designates samples that were not tested, and \* indicates that the histology, status, or expression is unknown.

NSCLC lines	Phospho-S473 status		Tumor histology	p53 status	Rb expression	Kras status
	HS	LS				
H1703	+	+	SQ	Mut	WT	WT
H157	+	+	SQ	WT	*	Mut
A549	+	–	SQ	WT	WT	WT
H1155	+	+	LC	Mut	Mut	Mut
H1355	–	–	AD	Mut	WT	Mut
H1334	+	+	LC	Mut	WT	WT
H460	+	+	LC	WT	WT	Mut
H1299	+	+	LC	Mut	WT	Mut
H727	+	+	CA	Mut	WT	Mut
H838	+	+	AD	WT	WT	WT
H2228	+	+	AD	WT	Mut	WT
H2594	+	+	ME	*	WT	*
H2444	–	–	*	*	WT	*
H2009	+	+	AD	Mut	*	Mut
H2409	+	+	AD	*	WT	*
H3072	+	+	*	*	WT	*
H2850	+	+	*	*	WT	*
H2973	+	+	*	*	WT	*
H1374	+	+	*	*	WT	*

in both the COOH terminus and in the activation domain, we analyzed five of the NSCLC cell lines for phosphorylation of both S473 and T308. Fig. 1A shows that the H1155, H157, and H1703 cells exhibit S473 phosphorylation under normal growth conditions and serum deprivation, but A549 and H1355 cells show little S473 phosphorylation in low-serum conditions. Levels of phosphorylated S473 were greatest in the H1155 cells. Regulation of S473 phosphorylation by serum withdrawal was only observed with the A549 cells. The response to IGF-I was greatest in the cells with the lowest levels of S473 phosphorylation (A549 and H1355).

Phosphorylation patterns for T308, the PDK1 site (Fig. 1B), generally correlated with the S473 pattern; only the H1155, H157, and H1703 cells displayed phosphorylation of Akt/PKB at T308. The cells with the highest S473 phosphorylation, H1155, displayed the highest levels of T308 phosphorylation in low-serum conditions. The other two lines that maintained high levels of S473 phosphorylation under serum starvation, H157 and H1703 cells, had different responses in terms of T308 phosphorylation. The H157 cells decreased T308 phosphorylation with serum starvation. The H1703 cells had very low levels of T308 phosphorylation, which increased with serum starvation. When the T308 immunoblot was exposed for longer periods of time, bands were clearly present in both lanes for the H1703 cells, but bands in the A549 and H1355 cells were never observed (data not shown). Interestingly, both H1703 and H1155 cells increased T308

phosphorylation when transitioned from normal growth conditions to serum deprivation, indicating that increased T308 phosphorylation might be an adaptive response to the stress of serum deprivation in these cells. Increased T308 phosphorylation has been observed with other stresses such as hypoglycemic shock by Ouyang *et al.* (66), who also showed discordant S473 and T308 phosphorylation. To determine whether discordant phosphorylation occurred in other NSCLC cell lines, we measured T308 phosphorylation in extracts from eight other NSCLC cell lines with constitutive S473 phosphorylation. We found that phosphorylation of T308 correlated with S473 phosphorylation in only four of eight cell lines (data not shown). In cell lines with T308 phosphorylation, there was a marked increase in T308 phosphorylation with serum deprivation, thereby lending support to the contention that T308 phosphorylation may be an adaptive response to serum starvation. The cells with the lowest S473 phosphorylation, A549 and H1355, did not exhibit T308 phosphorylation. Our immunoblotting data, therefore, predicted that three of five NSCLC cell lines would have fully active Akt/PKB.

Because the tumor suppressor, PTEN, regulates PDK1 and Akt/PKB activity through its lipid phosphatase function (67), we used immunoblotting to test whether Akt/PKB phosphorylation correlated with PTEN protein expression. Fig. 1C shows that, of three cell lines with phosphorylated Akt/PKB, two did not express PTEN protein. The absence of PTEN protein in H1155 and H157 cells is consistent with previous data showing nonsense mutations in both of these cell lines (68). Because H1703 cells showed both constitutive Akt phosphorylation and expression of PTEN protein, we sequenced PTEN cDNA from H1703 cells and found the sequence to be wild-type (data not shown). The biological basis for high levels of endogenous Akt/PKB phosphorylation in the face of wild type PTEN is unclear. In contrast to the low or absent PTEN in cells with phosphorylated Akt/PKB, the cell lines with the lowest levels of phosphorylated Akt had the highest level of PTEN protein expression (A549 and H1355). Both A549 and H1355 cells have wild-type PTEN (Ref. 69 and data not shown, respectively). Taken together, these data show that Akt/PKB phosphorylation and mutations in PTEN are inversely correlated in four of five NSCLC cell lines.

To determine whether multiple isoforms of Akt/PKB are expressed in these five NSCLC cell lines, we performed RT-PCR with isoform-specific primers and immunoblotting with commercially available isoform-specific antibodies. Fig. 1D shows that transcripts for all three Akt isoforms are ubiquitously expressed in each cell line. Fig. 1E shows that Akt 1 protein is expressed at similar levels in all five cell lines. In contrast, Akt 2 protein expression is lower in H157 and H1155 cells, and Akt 3 levels are lower in the A549 and H1355 cells, which have the lowest levels of Akt phosphorylation, respectively. Because these antibodies were unable to selectively immunoprecipitate individual Akt isoforms, we were unable to assign Akt activity to a specific isoform(s) (data not shown).

To demonstrate that Akt/PKB phosphorylation was dependent on PI3-K activity, we tested the ability of two PI3-K inhibitors, LY294002 and wortmannin, to inhibit Akt/PKB phosphorylation. Fig. 2A shows that both LY294002 and wortmannin completely inhibited phosphorylation of S473 in the three NSCLC cell lines that maintain S473 phosphorylation under serum deprivation. Native Akt/PKB levels did not change. Similar results were obtained for T308 phosphorylation (Fig. 2B). Cells without S473 or T308 phosphorylation did not respond to LY294002 (A549 and H1355). We observed similar effects with wortmannin when the dose was reduced to 100 nM (data not shown). Because wortmannin may inhibit other kinases such as phospholipase A<sub>2</sub> (70), PI4-K (71), and kinases with PI3-K domains such as DNA-PK, ATM, and ATR (72, 73), we limited all additional studies with PI3-K inhibitors to

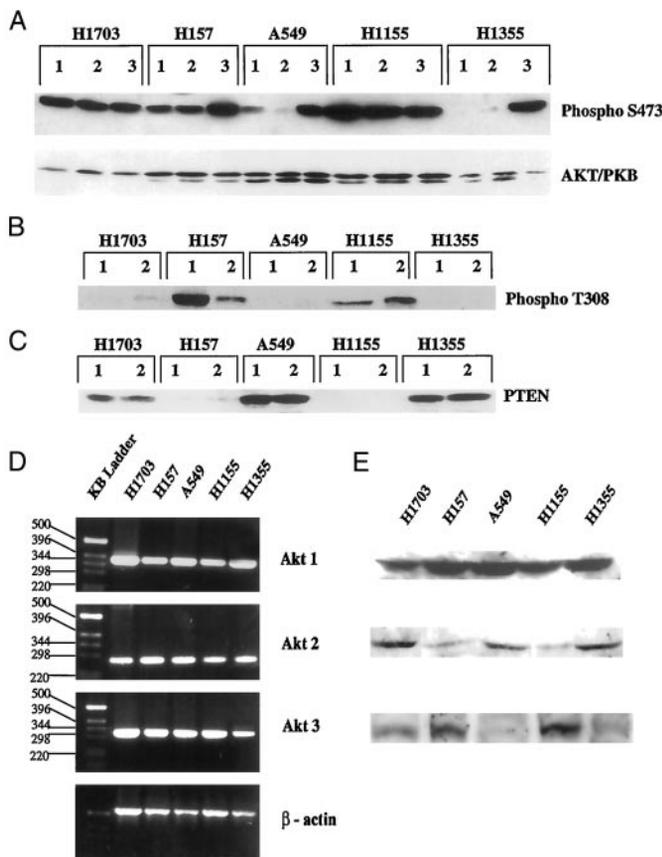


Fig. 1. Immunoblot analysis of Akt/PKB activity in five NSCLC cell lines. Cells were plated at  $1 \times 10^5$ /well and exposed to HS, LS, or LS with IGF-I stimulation for 10 min. Immunoblotting was performed as described. Phosphorylation of Akt/PKB at S473 is shown in the top panel in A. Total levels of Akt/PKB protein levels are shown in the bottom panel in A. Phosphorylation of T308 is depicted in B. PTEN protein expression is shown in C. Lane 1, HS; Lane 2, LS; Lane 3, LS + IGF-I (50ng/ml). A representative experiment from three independent experiments is shown. D, results of RT-PCR performed with isoform-specific Akt primers. The specificity of the reaction was confirmed by not observing a band when primers were omitted from the reaction mixture (data not shown). E, results of immunoblotting with commercially available, isoform-specific Akt antibodies.

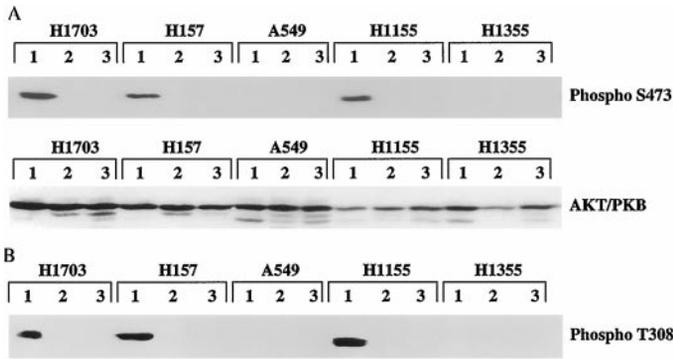


Fig. 2. LY294002 and Wortmannin inhibit phosphorylation of S473 and T308. Cells were plated at  $1 \times 10^5$ /well and placed in LS overnight. PI3-K inhibitors (LY294002 at  $25 \mu\text{M}$  or Wortmannin at  $1 \mu\text{M}$ ) were added 2 h before preparation of cell lysates. Immunoblotting was performed as described. Phosphorylation of Akt/PKB at S473 (A) and T308 (B) is evident in the three cell lines that have the highest levels of Akt/PKB activity (H1703, H157, and H1155). Basal levels of Akt/PKB are shown in the middle panel. Lane 1, LS; Lane 2, LS + LY294002; Lane 3, LS + Wortmannin. A representative experiment from three independent experiments is shown.

LY294002. (LY294002 may also have nonspecific effects; but only inhibition of the mammalian target of rapamycin, an Akt/PKB substrate (74), by LY294002 has been reported at doses used in these experiments (75). These data demonstrate that Akt/PKB phosphorylation in the three positive cell lines is PI3-K-dependent, making it unlikely that other kinases are directly involved in activating Akt/PKB.

We then performed *in vitro* kinase assays using glycogen synthase kinase-3 $\alpha/\beta$  (GSK-3 $\alpha/\beta$ ) as a substrate to demonstrate that phosphorylated Akt/PKB is enzymatically active. Fig. 3 shows that the highest levels of Akt/PKB activity are found in the H1155, H157, and H1703 cells, which have highest levels of phosphorylated Akt/PKB. The H1703 cells, which induced T308 phosphorylation with serum deprivation, slightly increased Akt/PKB activity under these conditions. Conversely, the H157 cells, which showed decreased T308 phosphorylation with serum deprivation, exhibited less kinase activity in low serum. Interestingly, cells that do not display phosphorylated Akt/PKB (A549 and H1355) contain small amounts of active Akt/PKB that can be detected once Akt/PKB is immunoprecipitated. Because LY294002 did not decrease the small amounts of Akt/PKB activity in these cells, Akt/PKB may be activated in these cells in a PI3-K-independent manner. The presence of active Akt/PKB in H1155, H157, and H1703 cells was also confirmed by detecting phosphorylated p70S6K, a kinase downstream of Akt/PKB, in these cell lines (data not shown). Similar to inhibition of Akt/PKB phosphorylation, LY294002 decreased Akt/PKB kinase activity most in cells with active Akt/PKB. Relative inhibition of Akt/PKB activity by LY294002 was greatest in the H1703 cells (58%), H157 (75%), and H1155 cells (58%). The less complete inhibition by LY294002 in the H1703 cells and H1155 cells may reflect enhanced metabolism of LY294002, insufficient time for inhibition of Akt/PKB activity, or the presence of Akt/PKB that is activated in a PI3-K-independent manner. When combined, the data from the phosphorylation and kinase assay experiments show that constitutive activation of Akt/PKB is PI3-K dependent in NSCLC cells.

**Akt/PKB Promotes Survival of NSCLC Cells.** To demonstrate that active Akt/PKB promotes cellular survival of NSCLC cell lines, we used pharmacological and genetic approaches to alter Akt/PKB activity and assess apoptosis. After treating NSCLC cells containing active Akt/PKB with LY294002 for 48 h, typical morphological changes of apoptosis were observed, including membrane blebbing, increased refractoriness, and chromatin condensation (data not shown). Other evidence for LY294002-induced

apoptosis included increased annexin V binding to cells as assessed by flow cytometry (data not shown). To measure apoptosis quantitatively, we assessed sub2N DNA formation by staining cellular DNA with propidium iodide before analysis using flow cytometry. Fig. 4 shows that apoptosis was potentiated 4–7-fold in the 3 cell lines that have the highest levels of active Akt/PKB (H157, H1703, and H1155), but LY294002 had only modest effects (2-fold induction) in the cells with the lowest levels of Akt/PKB activity (A549 and H1355). The small increases in apoptosis observed with LY294002 in A549 and H1355 cells may reflect nonspecific effects or inhibition of the small amount of Akt/PKB activity detected in the kinase assays over the course of 48 h. Compared with H1155 and H1703 cells, where the quantitative effects of LY294002 were similar in kinase and apoptosis assays, LY294002 exerted a smaller effect on apoptosis compared with kinase inhibition in the H157 cells. This may be related to different types of

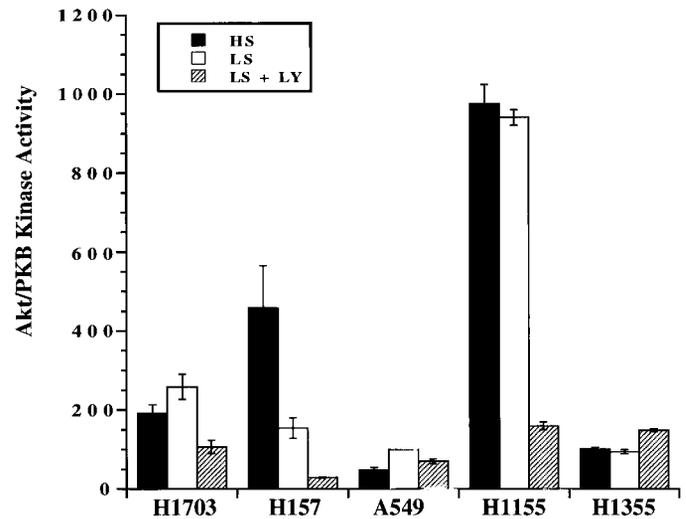


Fig. 3. Effect of serum deprivation and LY294002 on Akt/PKB kinase activity. Equal cell numbers were plated and allowed to attach for 24 h. Media were then changed to HS, LS, or LS with LY294002 ( $25 \mu\text{M}$ ) added for 2 h. Cells were harvested, and kinase activity was determined as described. Quantification of band density was performed using NIH Image software. Densitometry was performed over a range of exposures to ensure that relative differences in kinase activity were consistent. Bars, means  $\pm$  SE from five independently performed experiments. Black bars, HS; gray bars, LS; white bars, LS + LY294002.

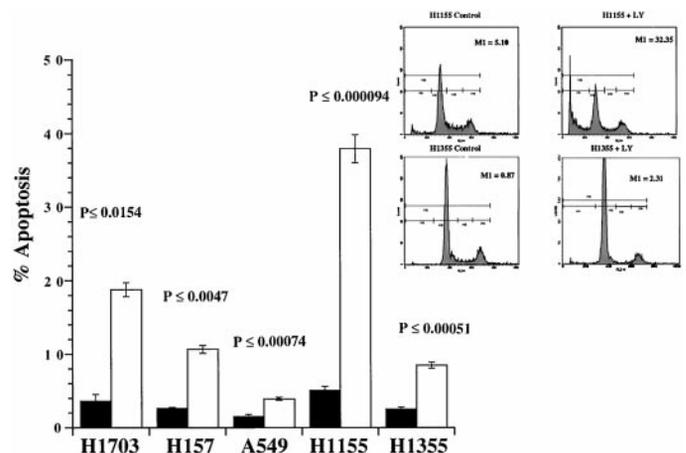


Fig. 4. LY294002 induces NSCLC apoptosis. Cells were plated at  $1 \times 10^5$ /well and were placed in 0.1% FBS with (gray bars) or without (black bars)  $25 \mu\text{M}$  LY294002 for 48 h. Cells were harvested, and apoptosis was quantified via flow cytometry as described. Bars, means  $\pm$  SD of a representative experiment done in triplicate from three independently performed experiments. Ps comparing LY294002 to control are shown above the data.

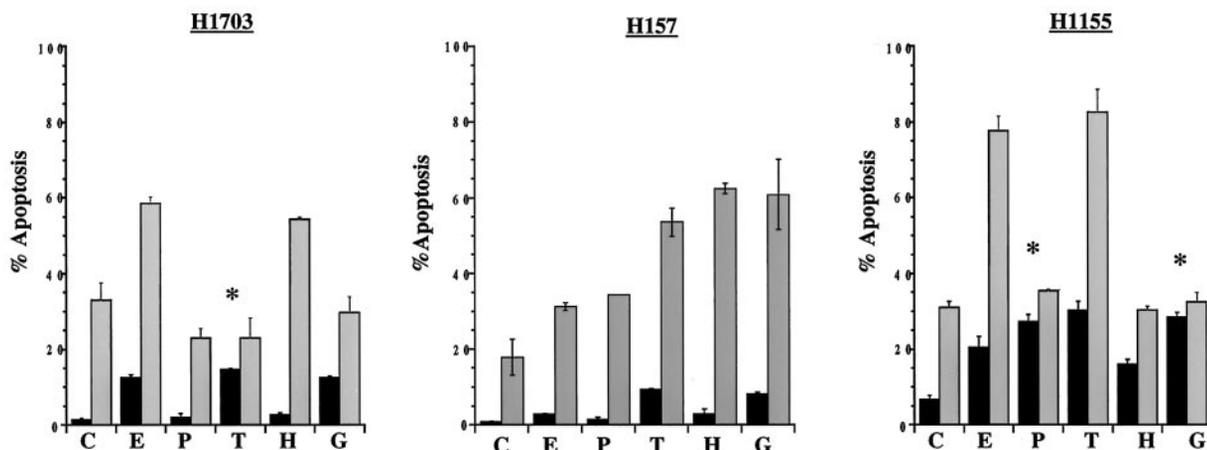
cellular adaptation over the course of the different experiments (2 h for kinase assay and 48 h for apoptosis) and may reflect the induction of PI3-K, Akt/PKB, or other survival pathways over 48 h in the H157 cells. Combined, LY294002 not only inhibits Akt/PKB phosphorylation and Akt/PKB activity, but also increases NSCLC cell apoptosis in relative proportion to the level of endogenous Akt/PKB activity.

**Modulation of Akt/PKB Activity Alters Sensitivity of NSCLC Cells to Chemotherapy.** Because LY294002 decreased NSCLC cell survival and NSCLC cells are known to be resistant to chemotherapy-induced apoptosis, we added LY294002 and individual chemotherapy agents to NSCLC cell lines and measured apoptosis. Doses of chemotherapy were chosen that caused minimal amounts of apoptosis based on dose-response curves performed in all five cell lines (data not shown). Fig. 5A shows that in the three cell lines with high Akt/PKB activity, basal levels of apoptosis were potentiated 6–16-fold by LY294002 (*Lanes 1*). Of the five lines, the H1155 cells, which have the highest levels of Akt/PKB, were the most sensitive to chemotherapy, indicating that endogenous Akt/PKB activity alone is not predictive for chemotherapeutic resistance. Nonetheless, Akt/PKB activity in H1155 cells clearly contributed to chemotherapeutic resistance, because the combination of LY294002 increased chemotherapy-induced apoptosis, especially in combination with etoposide and paclitaxel. The increased relative sensitivity of the H1155 cells to

chemotherapy may be related to the fact that they are the only cell line we tested that grows in suspension. Responses to individual agents and LY294002 were cell line-specific. On the basis of fractional inhibition analysis (63), synergy with LY294002 was apparent with all agents tested, including etoposide (*Lanes 2–3* of three lines), CDDP (*Lanes 3–2* of three lines), paclitaxel (*Lanes 4–2* of three lines), trastuzumab (*Lanes 5–2* of three lines), and gemcitabine (*Lanes 6, H157* cells only).

In cells lines with low levels of Akt/PKB activity (Fig. 5B), LY294002 increased apoptosis above baseline (*Lanes 1*), but the absolute level of apoptosis was much lower than that observed with the cell lines with high levels of Akt/PKB. Notably, apoptosis induced by chemotherapeutic agents alone in these cells was minimal, indicating that pathways other than Akt/PKB contribute to therapeutic resistance in A549 and H1355 cells. By fractional inhibition analysis, LY294002 was additive to all chemotherapies in H1355 cells except etoposide, where synergy was observed. In A549 cells, which have higher levels of Akt/PKB than the H1355 cells (see Fig. 3), synergy with etoposide, CDDP, Herceptin, and gemcitabine was noted. Overall, the effects of LY294002 were greatest with etoposide in all NSCLC cell lines, independent of endogenous Akt/PKB levels. These studies show that the level of endogenous Akt/PKB activity was predictive for quantifying response to LY294002 when used alone and with chemotherapy. Given that 94% of NSCLC cell lines we tested

### High levels of Akt/PKB activity



### Low levels of Akt/PKB activity

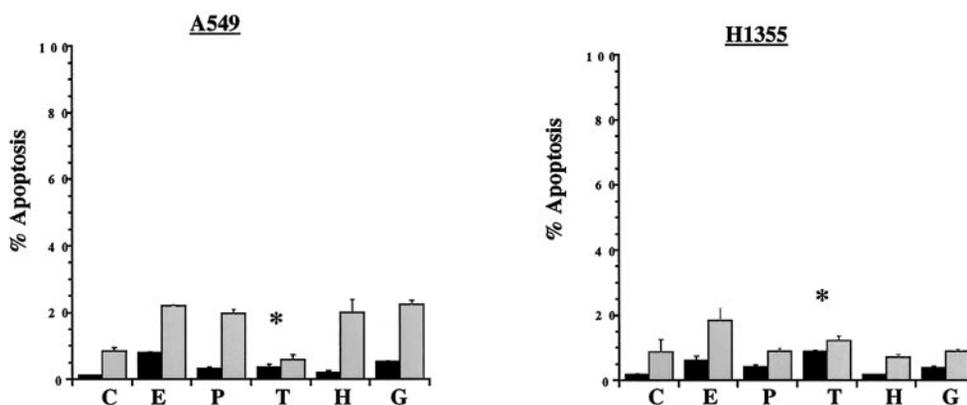


Fig. 5. LY294002 increases sensitivity of NSCLC cells to chemotherapy. Cells were plated at  $1 \times 10^5$ /well and placed in 0.1% FBS. LY294002 and chemotherapeutic agents were added simultaneously, and cells were incubated for 48 h. Apoptosis was quantified as described. Lane C, no chemotherapy; Lane E, etoposide (50  $\mu\text{M}$ ); Lane P, CDDP (20  $\mu\text{M}$ ); Lane T, paclitaxel (2.5  $\mu\text{M}$ ); Lane H, trastuzumab (10.5  $\mu\text{M}$ ); Lane G, gemcitabine (100  $\mu\text{M}$ ). Black bars, control; gray bars, LY294002 (25  $\mu\text{M}$ ). Bars, means  $\pm$  SD of a representative experiment done in triplicate from five independently performed experiments for each cell line. P's comparing LY294002 to control were  $<0.05$  unless indicated (\*).

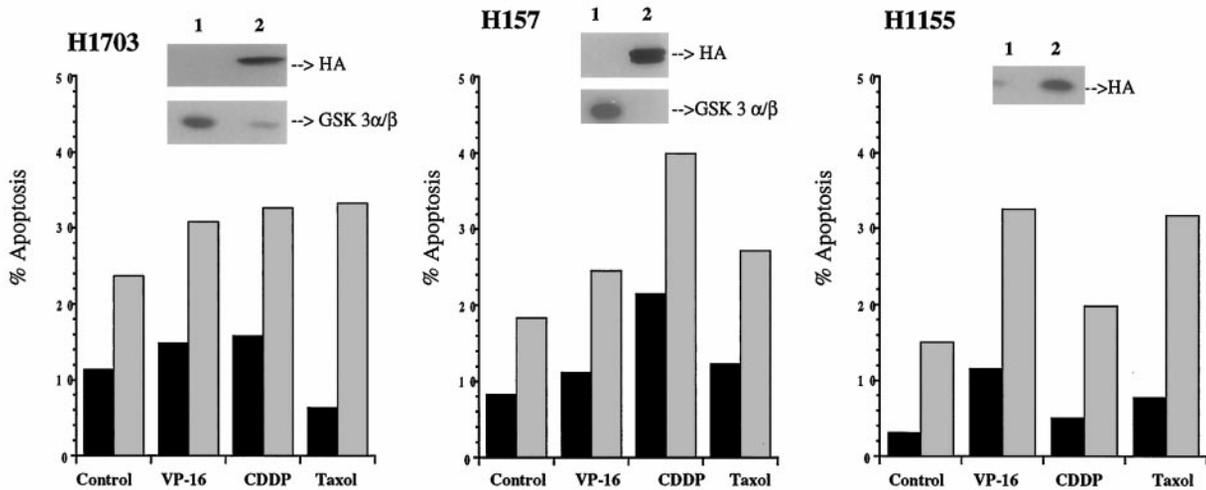
have constitutively active Akt/PKB, Akt/PKB activation may be a common mechanism that could be exploited to increase the effectiveness of chemotherapy.

To demonstrate that the effects of LY294002 and chemotherapy on NSCLC apoptosis were specific for inhibiting Akt/PKB activity, we transiently cotransfected NSCLC cell lines with HA-tagged dominant negative Akt/PKB (K179M Akt/PKB) and GFP and assessed apoptosis in the GFP-positive cells. Fig. 6 shows that in the cell lines with high Akt/PKB activity, transfection of K179M Akt/PKB resulted in a 2–5-fold increase in basal apoptosis. Basal levels of apoptosis were similar in GFP-positive and -negative cells, and basal apoptosis was higher in these experiments compared with earlier experiments because of nonspecific toxic effects of the lipid transfection reagent. Insets show the expression of HA-tagged K179M Akt/PKB (*top inset*, Lane 2) versus vector alone (*top inset*, Lane 1), as well as inhibition of Akt/PKB kinase activity (*bottom inset*, Lanes 1, vector alone, and Lanes 2, K179M Akt/PKB; not shown for H1155 cells). Adding chemotherapy to H1703, H157, and H1155 cells transiently transfected with K179M Akt/PKB potentiated apoptosis in all three cell lines. The additive effects on apoptosis were observed with K179M Akt/PKB and etoposide (Lanes 2), CDDP (Lanes 3), and paclitaxel (Lanes 4). The cells with the largest induction of apoptosis with K179M Akt/PKB, the H1155 cells, were also the cells most sensitive to LY294002. Quantitative differences in apoptosis between cells treated with LY294002 and chemotherapy (Fig. 5) and K179M Akt/PKB and chemotherapy (Fig. 6) were possibly a result of conservative

gating (*i.e.*, gating only on strongly GFP-positive cells) or a shorter incubation time with chemotherapy for the transfected cells (24 h) versus the cells treated with LY294002 (48 h). Unfortunately, extending transfection experiments for an additional 24 h resulted in significantly lower levels of GFP expression, which limited our ability to gate on transfected cells. In contrast to the potentiation of apoptosis by K179M Akt/PKB in cells with high Akt/PKB activity, transfection of A549 cells with K179M Akt/PKB had little effect on basal levels of apoptosis or etoposide-induced apoptosis, although expression of HA-tagged K179M Akt/PKB was easily detected (*inset*). These studies confirm and extend the data generated with LY294002 by demonstrating that inhibition of Akt/PKB with either LY294002 or K179M Akt/PKB selectively increases apoptosis in cells with high levels of active Akt/PKB.

We then performed the converse experiments: we transiently transfected NSCLC cells with low Akt/PKB activity with constitutively active Akt/PKB to determine whether increasing Akt/PKB activity would attenuate chemotherapy-induced apoptosis. Fig. 7 shows that transfection of A549 or H1355 cells with HA-tagged Myr Akt/PKB resulted in expression of the HA epitope tag (*top insets*), and increased Akt/PKB activity (*bottom insets*). Transfection of Myr Akt/PKB had no protective effect on basal levels of apoptosis, indicating that these cells activate other pathways for survival.<sup>3</sup> In cells transfected with vector alone, etoposide increased apoptosis by 257% in A549 cells and 211% in H1355 cells. In cells transfected with Myr Akt/PKB, etoposide increased apoptosis by only 171% in the A549 cells and

### High levels of Akt/PKB activity



### Low levels of Akt/PKB activity

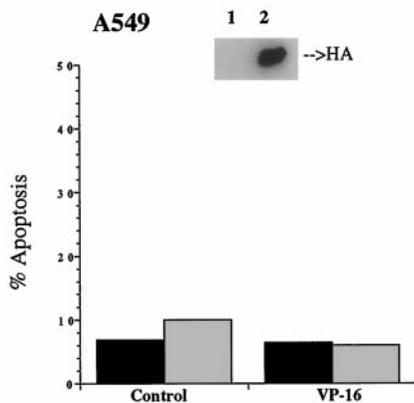


Fig. 6. Dominant negative Akt/PKB increases apoptosis and sensitizes cells to chemotherapy. Cells were plated with at  $2 \times 10^5$ /well, and parallel samples were transiently cotransfected with plasmids encoding GFP and pSR $\alpha$  (black bars) or GFP and K179M Akt/PKB (gray bars) as described. Cells were allowed to recover from transfection for 24 h, then exposed to LS  $\pm$  chemotherapy for 24 h. Samples were harvested for immunoblotting with anti-HA antibodies and for *in vitro* kinase activity against GSK $\alpha/\beta$ . Samples were also fixed, stained with propidium iodide, and prepared for flow cytometry. Apoptosis was quantified by gating on GFP-positive cells and performing cell cycle analysis. Lane 1, pSR $\alpha$ ; Lane 2, K179M Akt/PKB. A representative experiment from three independent experiments performed with each cell line is shown.

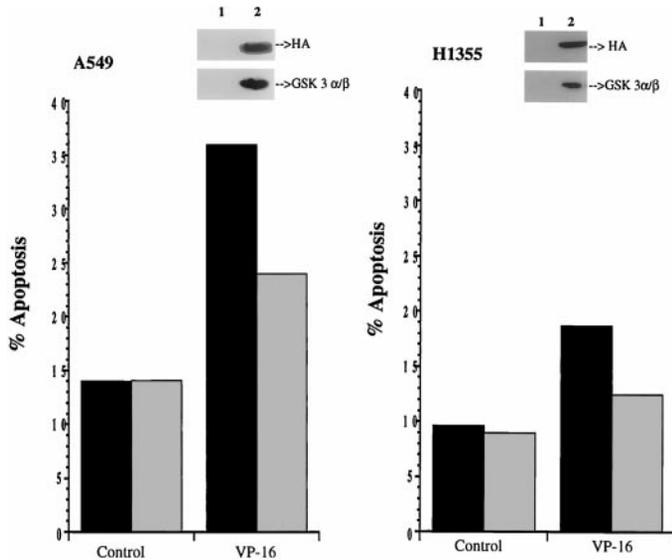


Fig. 7. Constitutively active Akt/PKB increases chemotherapeutic resistance of NSCLC cells with low levels of Akt/PKB activity. A549 and H1355 cells were plated at  $2 \times 10^5$ /well, and parallel samples were transiently cotransfected with plasmids encoding GFP and pSR $\alpha$  (black bars) or GFP and Myr Akt/PKB (gray bars) as described. Cells were allowed to recover from transfection for 24 h, then exposed to LS  $\pm$  chemotherapy for 24 h. Samples were harvested for immunoblotting with anti-HA antibodies and for *in vitro* kinase activity against GSK $\alpha/\beta$ . Samples were also fixed, stained with propidium iodide, and prepared for flow cytometry. Apoptosis was quantified by gating on GFP-positive cells and performing cell cycle analysis. Lane 1, pSR $\alpha$ ; Lane 2, Myr Akt/PKB. A representative experiment from three independent experiments is shown for each cell line.

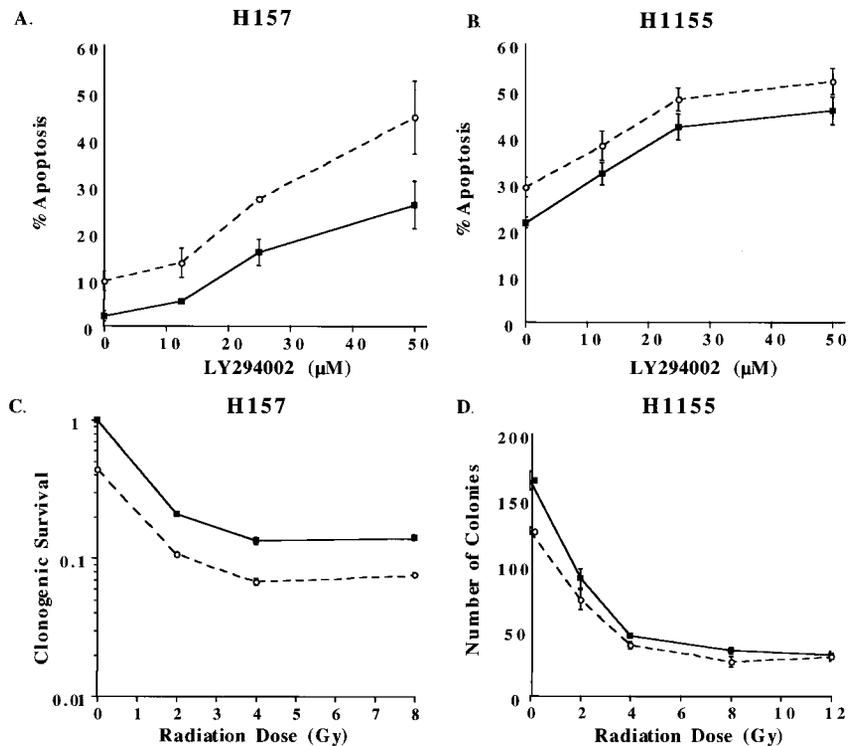
133% in H1355 cells, demonstrating that increasing Akt/PKB activity increases chemotherapeutic resistance, even in cells already resistant to chemotherapy. These studies with Myr Akt/PKB complement the studies of K179M Akt/PKB in cells with high Akt/PKB activity by showing that increasing the activity of Akt/PKB in cell lines that exhibit little endogenous activity results in greater cell survival when cells are exposed to chemotherapy. The fact that two opposite ap-

proaches to modulating Akt/PKB activity yielded opposite, predictable responses in terms of basal apoptosis and sensitivity to chemotherapy emphasizes the importance of the Akt/PKB pathway in NSCLC survival and chemotherapeutic resistance.

**Modulation of Akt/PKB Activity Alters Sensitivity of NSCLC Cells to Radiation.** To test whether Akt/PKB promoted resistance to irradiation-induced NSCLC cell apoptosis, we used LY294002 or K179M Akt/PKB in combination with radiation. Fig. 8, A and B, shows that when H157 and H1155 cells were exposed to 6 Gy in the absence of LY294002, apoptosis was increased from 2–10% (H157) and 21–29% (H1155). When cells were irradiated in the presence of LY294002, apoptosis increased in a dose-dependent manner, but the relative increase in apoptosis with 6 Gy in the presence of LY294002 was the same as for LY294002 alone. Therefore, the effects of LY294002 on irradiation-induced apoptosis appeared additive. Because the lethal effects of irradiation often require cell cycle progression, so-called “replicative cell death” (76), clonogenic assays of H157 and H1155 were performed. H157 and H1155 cells received increasing doses of radiation in the absence or presence of LY294002 and then were split to allow colony growth. Panels C and D confirm the radioresistance of these cells in that the number of cell colonies decreased by only  $\sim 1$  log after 8 Gy (H157 cells) or 12 Gy (H1155 cells). LY294002 decreased the number of colonies at 8 days by 25% (H1155 cells) and 55% (H157 cells) without irradiation, but had no additional effect on colony formation as the irradiation dose was increased. Therefore, LY294002 did not appear to increase replicative cell death. The effects of LY294002 on clonogenic growth appeared to be additive to the effects with radiation. These data suggest that the mechanism of LY294002 potentiation of irradiation-induced death is not attributable to effects on replicative cell death, but rather to apoptosis. The fact that LY294002 did not significantly change the shoulder of the dose-response curve (*i.e.*, did not decrease the slope of the curve at low doses such as 2 Gy) suggested that LY294002 did not affect the cells’ ability to repair DNA damage.

To confirm that the effects of LY294002 on potentiating irradiation-

Fig. 8. LY294002 increases radiation-induced apoptosis and decreases radiation-induced clonogenic growth in NSCLC cells with active Akt/PKB. Cells were plated at  $1 \times 10^5$ /well and placed in LS after attachment. LY294002 was added at the indicated doses (A, H157 cells; B, H1155 cells), and cells were immediately irradiated with 8 Gy. Cells were incubated for 48 h, and apoptosis was quantified as described. —, control samples; ---, irradiated samples. Clonogenic growth with H157 cells (C) and H1155 cells (D) was assessed in the absence (—) or presence (---) of LY294002 as described. Data points are the means  $\pm$  SD of a representative experiment done in triplicate from three independently performed experiments.



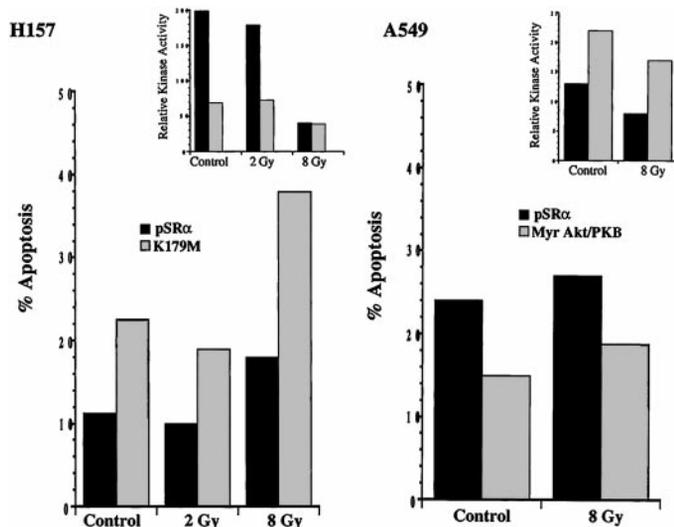


Fig. 9. Modulating Akt/PKB activity with kinase-dead Akt/PKB or constitutively active Akt/PKB alters the sensitivity of NSCLC cells to radiation. H157 and A549 were plated with at  $2 \times 10^5$ /well, and parallel samples were transiently cotransfected with plasmids encoding GFP and pSR $\alpha$  (black bars), GFP and K179M Akt/PKB (gray bars, H157 cells), or GFP and Myr Akt/PKB (gray bars, A549 cells) as described. Cells were allowed to recover from transfection for 24 h, then exposed to LS  $\pm$  irradiation for 24 h. Whole cell lysates from ungated samples were harvested for immunoblotting with anti-HA antibodies (not shown) and for *in vitro* kinase activity against GSK $\alpha/\beta$  (insets). Samples were also fixed, stained with propidium iodide, and prepared for flow cytometry. Apoptosis was quantified by gating on GFP-positive cells and performing cell cycle analysis. A representative experiment from three independent experiments is shown for each cell line.

tion-induced apoptosis were attributable to Akt/PKB inhibition, we transiently cotransfected H157 cells with K179M Akt/PKB and GFP or vector and GFP, irradiated the cells, and measured apoptosis of GFP-positive cells. Fig. 9A (inset) shows that, compared with cells transfected with vector alone, unirradiated cells transfected with K179M Akt/PKB had 66% less Akt/PKB activity compared with controls (inset; HA expression not shown). Interestingly, a dose-dependent, irradiation-induced decrease in Akt/PKB activity was observed ( $>80\%$  with 8 Gy; inset, black bars). The administration of 8 Gy to cells transfected with K179M Akt/PKB increased apoptosis 2-fold over cells transfected with vector alone. A similar approach was taken to transfect A549 cells with Myr Akt/PKB. A549 cells were transfected with Myr Akt/PKB and GFP or vector alone and GFP and exposed to 8 Gy, and apoptosis was measured 48 h later. Fig. 9B (inset) shows that the inhibition of kinase activity caused by irradiation alone (black bars) was more modest with the A549 cells (37%) compared with the H157 cells. Myr Akt/PKB increased kinase activity 84% above baseline, and this difference was maintained when the cells were irradiated. Myr Akt/PKB decreased basal levels of apoptosis, and the differences in apoptosis between Myr Akt/PKB and vector-containing cells were maintained when the cells were irradiated. The discrepancy between Myr Akt/PKB inhibiting basal levels of apoptosis under these conditions [as opposed to conditions of the chemotherapy experiments (Fig. 7)] may be related to the fact unirradiated A549 cells were left of the tissue culture incubator to control for the conditions the irradiated cells were exposed to when taken to the linear accelerator. The change in CO<sub>2</sub> content and/or temperature may therefore have provided a stress to the unirradiated, transfected A549 cells that Myr Akt/PKB protected against. Together, these data show that pharmacological or genetic approaches that decrease Akt/PKB activity potentiate irradiation-induced apoptosis. Moreover, this highlights the importance of Akt/PKB as a survival kinase whose activity confers protection against both chemotherapy- and irradiation-induced apoptosis in NSCLC cells.

## DISCUSSION

These experiments are the first to identify a serine/threonine kinase, Akt/PKB, as a crucial mediator of NSCLC cell survival and therapeutic resistance. We used immunoblotting with phospho-specific antibodies to demonstrate constitutive Akt/PKB activity in 16 of 17 cell lines, and then confirmed these observations with *in vitro* kinase assays performed on a smaller subset of these cell lines. Because these phospho-specific antibodies recognize analogous sites to S473 and T308 in Akt2/PKB $\beta$  and Akt3/PKB $\gamma$ , and because we were unable to selectively immunoprecipitate individual Akt isoforms with commercially available antibodies, we were unable to assign Akt activity to different Akt/PKB isoforms. Nonetheless, expression of Akt3 protein appears to correlate most closely with the results of kinase assays performed in these cell lines. Reactivity with the phospho-specific antibodies correlated strongly with *in vitro* kinase activity, which suggests that these antibodies may have utility in measuring Akt activity in human tumors. How do NSCLC cells maintain Akt/PKB activity? Possible explanations include production of an autocrine GF, activation of various GFRs, mutations of PI3-K or other upstream kinases, loss of regulatory phosphatases such as PTEN or SHIP, or mutations in Akt itself. Although the mechanism of Akt/PKB activation in NSCLC cells is unclear, it is likely posttranslational. This is supported by observations that gene amplification of the three Akt/PKB isoforms is infrequent in NSCLC cells and tissues, and that total Akt/PKB protein levels did not change in our experiments. Possible mechanisms for constitutive Akt/PKB activation include activation of upstream kinases such as PI3-K or PDK1, and/or inhibition of lipid or protein phosphatases that normally regulate Akt/PKB function.

The ability of PI3-K inhibitors to decrease Akt/PKB phosphorylation and activity suggest that the activation of Akt/PKB is PI3-K-dependent, although we have not directly assayed PI3-K activity in these cells. Kinases or GFRs upstream from PI3-K could also be activated and responsible for Akt/PKB activation, but no correlation between *K-ras* status and Akt/PKB activity was noted. Increased PDK1 activity, which depends on PI3-K activity and results in T308 phosphorylation, may explain Akt/PKB activation in NSCLC cells. This is supported by the fact two of three NSCLC cell lines with active Akt/PKB (H157 and H1155) altered T308 phosphorylation without detectable PTEN. Although PDK1 activity was thought previously to be constitutive, recent reports demonstrate that PDK1 activity can be increased by cellular stresses such as oxidative stress and sphingomyelinase activation (77, 78). It is unknown if the stress of serum deprivation in these studies would have a similar effect in activating PDK1 in these cells. Additional support for the involvement of PDK1 in NSCLC Akt/PKB activation is that a novel isoform of the PKC family is a substrate for PDK1 and is also constitutively active in these cells and promotes NSCLC cell survival.<sup>4</sup>

Aberrant phosphatase function may also contribute to Akt/PKB activation in NSCLC cells. The lipid phosphatases PTEN and SHIP have been shown to regulate Akt/PKB activity. PTEN is a tumor suppressor and functions as a 3' phosphoinositide phosphatase to regulate both PDK1 and Akt/PKB activity. The *PTEN* gene is wild-type in three of five NSCLC cell lines, one of which has high levels of Akt/PKB and two of which have the lowest levels of Akt/PKB activity (A549 and H1355). Although T308 phosphorylation was not apparent in the A549 and H1355 cells, some S473 phosphorylation was observed (Fig. 1), which conflicts with published reports showing that PTEN regulates phosphorylation of both sites. Surprisingly, the H1703 cells expressed lower levels of PTEN expression, had wild-type PTEN, and had the second-highest levels of Akt/PKB activity

<sup>4</sup> A. Clark and P. Dennis, manuscript in preparation.

(Fig. 3). This suggests a possible dose effect of PTEN protein on Akt/PKB activity or the activation of upstream kinases that can bypass or overwhelm the capacity of PTEN to regulate Akt/PKB activity. Of note, PTEN regulation of Akt/PKB is complicated further by the demonstration that PTEN function is regulated by phosphorylation (79). In an attempt to further correlate PTEN expression with S473 phosphorylation, we analyzed 10 other NSCLC cell lines from Table 1 that exhibited S473 phosphorylation, and we found that 9 of 10 lines expressed PTEN that migrated normally (data not shown). Although we cannot rule out missense mutations in these nine cell lines, these data, together with the findings that *PTEN* gene mutations are infrequent in NSCLC specimens and cell lines (68, 69, 80–82), question the importance of PTEN in regulating Akt/PKB activity in NSCLC cells.

SHIP is another lipid phosphatase that regulates Akt/PKB activity by dephosphorylating PIP-3 at the 5' position; but because this effect appears to be restricted to hematopoietic cells and tissues, we feel that SHIP is unlikely to be involved in the regulation of Akt/PKB activity in NSCLC cells (83, 84). A phosphatase more likely to be involved in Akt/PKB regulation in NSCLC cells is the protein phosphatase, PP2A, which regulates Akt/PKB function by directly dephosphorylating S473 and T308 (85). Of note, PP2A is inactivated under conditions of cellular stress (86), and a subunit of PP2A, PPP2R1B, is a putative tumor-suppressor gene in lung cancer (87). We are currently testing for allelic loss or mutations in PPP2R1B as well as evaluating changes in PP2A function with serum deprivation in NSCLC cells with active Akt/PKB.

In addition to showing that Akt/PKB is constitutively active in most NSCLC cells, we used two approaches to inhibit Akt/PKB to demonstrate that Akt/PKB promotes chemotherapeutic resistance. The first approach used two commercially available small molecules. LY294002 and wortmannin inhibited both sites of Akt/PKB phosphorylation, inhibited kinase activity, and increased NSCLC apoptosis in proportion to inhibition of Akt/PKB activity. Although these experiments were performed under conditions of serum deprivation, we obtained similar results in 10% FBS when the LY294002 dose was increased or given more frequently (data not shown). This is consistent with the reversible nature of PI3-K inhibition by LY294002 and the induction of PI3-K activity by GFs contained in 10% FBS. We chose to perform our experiments under conditions of serum deprivation because, after 48 h of serum deprivation, 5–10% of cells were found to be in S-phase (data not shown), which approximates the number of cells within solid tumors thought to be cycling at any given time. Similar to its selective effect on increasing apoptosis in NSCLC cells with active Akt/PKB, LY294002 had the greatest effects on chemotherapy-induced apoptosis in cells with the highest levels of Akt/PKB activity. The effect of combining LY294002 with chemotherapy did not appear to depend on the mechanism of activity of the chemotherapeutic agents, as a DNA-damaging agent, (CDDP), a topoisomerase II-inhibitor (etoposide), a taxane (paclitaxel), a nucleoside analogue (gemcitabine), and an erbB-2 inhibitor (Trastuzumab) could all be effectively combined with LY294002 to increase apoptosis. Interestingly, the most effective combination of LY294002 with chemotherapy was observed with etoposide, as synergy was observed in all five NSCLC cell lines tested. A mechanistic connection between Akt/PKB function and topoisomerase II function is not apparent. Although one other report has shown that LY294002 increases chemotherapy-induced apoptosis, these authors used higher doses of LY294002 commonly associated with nonspecific effects in only one leukemia cell line, HL60, that is known to be very sensitive to apoptotic stimuli. In addition, they did not use genetic approaches to demonstrate specificity for PI3-K (88). Therefore, this study is the first comprehensive demonstration that a PI3-K inhibitor could syn-

ergize with different chemotherapies in adherent, chemotherapeutically resistant cells.

The second approach we used was a genetic approach to transfect dominant negative Akt/PKB. These results with K179M Akt/PKB were similar to those observed with LY294002 and demonstrated that the effects of LY294002 were specific for Akt/PKB inhibition. Transient transfection of K179M Akt/PKB caused inhibition of Akt/PKB activity and increased chemotherapy-induced apoptosis selectively in cells with active Akt/PKB. Although the transfection efficiency ranged from 10–40% based on quantifying GFP-positive cells, we were able to demonstrate significant inhibition of kinase activity in lysates from entire plates, thus demonstrating a dominant/negative mutant effect of the kinase-dead Akt/PKB. Gating on GFP-positive cells allowed us to selectively assess cell cycle changes and apoptosis in transfected cells. To reconcile quantitative differences of LY294002 and K179M Akt/PKB on chemotherapy-induced apoptosis, we attempted to generate clones of H157 and H1703 cells stably transfected with K179M Akt/PKB, but no colonies grew in antibiotic selection, supporting further the importance of Akt/PKB in survival of these cells.

These same two approaches to inhibit Akt/PKB were also used to establish a role for Akt/PKB activity in cellular survival after irradiation. LY294002 was additive to the effects of radiation on potentiating apoptosis and inhibiting clonogenic growth. The effects of LY294002 on apoptosis were confirmed by combining radiation with dominant negative Akt/PKB in cells with high endogenous activity and demonstrating increased apoptosis with K179M Akt/PKB. LY294002 did not sensitize NSCLC cells to irradiation-induced inhibition of clonogenic growth, because when the data in Fig. 8 is replotted with elimination of the initial decrease in colony formation with LY294002 alone, the curves are virtually superimposable (data not shown). This finding is not consistent with published data that shows that both PI3-K inhibitors increase radiation sensitivity (72, 73, 89, 90). However, all of these studies used wortmannin or LY294002 at concentrations 2–50-fold greater than the concentrations used in our studies (concentrations that inhibit other kinases, including those involved in DNA repair after irradiation). A conservative interpretation of our radiation data would therefore suggest that Akt/PKB inhibition in combination with radiation is at least additive in induction of apoptosis and inhibition of clonogenic growth, but the effects of LY294002 on clonogenic growth may be more pronounced if the assays were to be performed with higher doses of LY294002.

Our results have important implications for subsequent preclinical and clinical studies. The fact that Akt/PKB promotes resistance to both chemotherapy and irradiation suggests that Akt/PKB activity might have predictive value for response to chemotherapy and/or irradiation, but only when combined with activities of other kinases known to promote cellular survival. Different kinase activities could then be combined to generate kinase profiles of human tumors. If the incidence of Akt/PKB activation in NSCLC cell lines (~90%) is indicative of the incidence of Akt/PKB activation in NSCLC tumors, then evaluation of Akt/PKB activity *in vivo* would be very important. However, evaluation of Akt/PKB as a predictive, or even prognostic factor, depends on the development of reliable assays to measure Akt/PKB activity. The fact that immunoblotting with phospho-specific antibodies correlated with *in vitro* Akt/PKB kinase assays suggests that utilization of these antibodies in standard pathological techniques such as immunohistochemistry and/or immunoblotting might allow valid surrogate measurements of Akt/PKB activity *in vivo*, and would thus facilitate assessment of Akt/PKB's clinical importance. Perhaps the most important implication of these studies is that approaches that target Akt/PKB might be useful clinically. We are currently evaluating novel small-molecule inhibitors designed to have

specificity for Akt/PKB. However, concerns about tumor specificity are often raised when molecules such as Akt/PKB are targeted that are present in normal cells and tumor cells. Our data with serum deprivation suggests the possibility that if tumor cells maintain Akt/PKB activity under stress such as serum deprivation (and untransformed cells do not), then tumor cells may have a dependence on Akt/PKB activity for survival that normal cells do not share. This dependence may be even greater *in vivo* because of stressors unique to tumor tissue, such as hypoxia, acidity, abnormal vascularization, and aneuploidy, and therefore this might be effectively exploited. Moreover, if approaches to inhibit Akt/PKB are developed clinically, clinical trials should be designed to include only those patients whose tumors possess Akt/PKB activity, as our data indicated that only cells with active Akt/PKB responded to the inhibition of Akt/PKB by pharmacological or genetic means.

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# Cancer Research

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## Akt/Protein Kinase B Is Constitutively Active in Non-Small Cell Lung Cancer Cells and Promotes Cellular Survival and Resistance to Chemotherapy and Radiation

John Brognard, Amy S. Clark, Yucheng Ni, et al.

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