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Inhibition of Akt/Protein Kinase B Signaling by Naltrindole in Small Cell Lung Cancer Cells

Yulong L. Chen, P. Y. Law, and Horace H. Loh

Department of Pharmacology, the University of Minnesota Medical School, Minneapolis, Minnesota

ABSTRACT

The phosphatidylinositol 3-kinase-Akt/protein kinase B (PKB) survival signaling is very important for cancer cell survival and growth. Constitutively active phosphatidylinositol 3-kinase-Akt/PKB signaling in small cell lung cancer (SCLC) is a major factor for the survival of SCLC cells. Inhibitors of this signaling pathway would be potential antitumor agents, particularly for SCLC. Here we report that naltrindole, which has been used as a classic δ opioid antagonist, inhibited growth and induced apoptosis in the three characteristic SCLC cell lines, NCI-H69, NCI-H345, and NCI-H510. Naltrindole treatment reduced constitutive phosphorylation of Akt/PKB on serine 473 and threonine 308 in cells. We found that the levels of constitutive phosphorylation of Akt/PKB on serine 473 correlate with the sensitivity of the three cell lines to naltrindole treatment. Furthermore, naltrindole treatment not only reduced the phosphorylation of the Akt/PKB upstream kinase phosphoinositide-dependent kinase-1, but also its downstream effectors glycogen synthase kinase-3β and the Forkhead transcription factors AFX and FKHR. DNA array analysis of 205 apoptosis-related genes indicated that some Akt/PKB-dependent genes were either up- or down-regulated by naltrindole. Flow cytometric and microscopic analyses clearly showed that naltrindole induced apoptosis in SCLC cells. RNA interference experiments confirmed that naltrindole-induced cell death was associated with the Akt/PKB survival pathway. Together, these results show that naltrindole is a new inhibitor of the Akt/PKB signaling pathway, suggesting that naltrindole could be a potential lead for the development of a new type of inhibitors that target the constitutively active Akt/PKB signaling-dependent SCLC cells.

INTRODUCTION

Lung and bronchus cancer is one of the deadliest cancers. In 2003, 157,200 people were projected to die of the disease in the United States (1). Small cell lung cancer (SCLC) accounts for approximately 25% of all lung cancers (2). Current treatments do not yield a satisfactory survival rate. In fact, approximately 90% of the patients die within 5 years after diagnosis (2). Malignant growth of SCLC cells may involve multiple genetic aberrations, including loss of the retinoblastoma (*RB*), p53, and $p16^{ink4A}$ tumor suppressor genes, overexpression of the *Bcl-2* and *Myc* family genes, and mutation of the oncogene *ras* (3).

Phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B, PKB) signaling has been found to be involved in the survival and proliferation of a variety of tumor cells (4), including SCLC (5, 6) and non–small-cell lung cancer (NSCLC) cells (7). PI3K is a heterodimer of the regulatory p85 and catalytic p110 subunits. A variety of growth factors can activate PI3K through activation of their cognate receptors, leading to the activation of Akt/PKB signaling (8). Akt/PKB is the cellular homologue of the product of the v-*akt* oncogene (9) and has three isoforms: Akt-1, -2, and –3 (or PKB-α, -β, and –γ; ref. 10). Akt/PKB mediates a variety of biological functions, including glucose uptake, protein synthesis, and inhibition of apoptosis (8). Akt/PKB can mediate cell survival and growth by regulating both post-translational mechanisms and gene transcription (11). The Akt/PKB kinase activity is regulated by phosphorylation on two regulatory sites, threonine 308 in the activation loop of the catalytic domain and serine 473 in the COOH-terminal regulatory domain (12). One of the Akt/PKB functions is to promote cell survival by inhibiting apoptosis, following the phosphorylation of several downstream targets, which include downstream effectors such as Bad, caspase-9, cAMP-response-element-binding protein, Forkhead transcription factors (AFX, FKHR), glycogen synthase kinase-3 (GSK-3), IκB kinase-α, nitric oxide synthase, and p70 ribosomal protein S6 kinase (13).

Akt/PKB kinase is dysregulated in numerous tumors by several different mechanisms, including overexpression of the proteins, constitutive activation in tumors harboring mutation *ras* oncogenes, or inactivation of an inhibitory phosphatase (4). Manipulation of Akt/PKB activity has resulted in altering the response of tumor cells to chemotherapy and irradiation (6, 7). Selective inhibitors of the PI3K/Akt signaling pathway are not only very potent tools for investigating biological roles of this important signaling pathway (14) but also potential therapeutic candidates for the treatment of PI3K/Akt signaling-dependent tumors such as SCLC (6) and NSCLC (7). Although there is tremendous interest in regulating the PI3K/Akt signaling pathway for the benefit of killing cancer cells (15, 16), not many inhibitors for this pathway have been published since the first synthetic PI3K inhibitor LY294002 was reported (14).

Naltrindole is a synthetic alkaloid compound designed and used as a classic δ-opioid receptor antagonist (17). Besides having antiopioid function, naltrindole has been reported to be a potent immunosuppressant in immune cells and in the transplant animal models (18, 19). In this study we report that naltrindole is a new inhibitor of the Akt/PKB signaling pathway. Naltrindole selectively inhibits the growth of SCLC cells. The inhibition of tumor cell growth by naltrindole correlates with the induction of apoptotic cell death and with the inhibition of the Akt/PKB survival cascade.

MATERIALS AND METHODS

Cell Culture and Reagents. The SCLC cell lines NCI-H69, NCI-H345, and NCI-H510 were purchased from American Type Culture Collection (Manassas, Virginia). SCLC cells were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified atmosphere at 37°C. [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]-enkephalin (DAMGO), [D-Pen^2,D-Pen^5]-enkephalin (DPDPE), morphine, etorphine, and naloxone were supplied by the National Institute on Drug Abuse. Naltrexone was purchased from DuPont Pharmaceuticals (Wilmington, DE). Naltrindole and other chemicals were purchased from Sigma (St. Louis, MO).

Cell Growth Assay by 3-(4,5-Dimethylthiazol-2-yl)-2,5-dibenzyltetrozolium bromide. On day 0, single SCLC cells were plated in 96-well plates at 10,000 cells/well in the corresponding medium and incubated at 37°C as described previously (20). Cells were treated at the indicated concentrations of drugs on day 0. On days 3 and 6, viable cells were determined with the

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Requests for reprints: Y. L. Chen, Department of Pharmacology, the University of Minnesota Medical School, 6–120 Jackson Hall, 321 Church St. S. E., Minneapolis, MN 55455. Phone: 612-626-6539; Fax: 612-625-8408; E-mail: chenx112@tc.umn.edu.

3-(4,5-dimethylthiazol-2-yl)-2,5-dibenzyltetrozolium bromide (MTT) assay according to the manufacturer's protocol (Promega, Madison, WI). Each datum point represents the mean ± SE of a representative experiment in either triplicates or sextuplicates.

Anchorage-Independent Clonogenic Assays. The procedure for the colony formation assay was described previously (21). In brief, single-cell suspensions of NCI-H69 were mixed with a 0.33% top soft agar mixture in RPMI 1640 containing 10% FBS and plated at a density of 1×10^5 cells per dish (35-mm diameter) over a 0.50% bottom agar layer in the same medium as used in the top agar layer. Both layers contained the indicated concentrations of naltrindole and naloxone. The cells were fed once on days 6, 11, and 16 with 0.5 mL of the same medium described above containing the indicated concentrations of naltrindole or naloxone. The colonies were fixed 21 days after the cells were plated. Colonies (>16 cells) were manually enumerated under a light microscope. Results were presented as the means ± SE from triplicate dishes.

Morphologic Analysis of Apoptosis. The method was adapted from the published procedure (21). Briefly, on day 0, cells were plated in 96-well plates at 10,000 cells/well in the corresponding medium containing the indicated concentrations of naltrindole and incubated at 37°C as described above. On day 3, the untreated cells (control) and drug-treated cells were fixed in 2.5% glutaraldehyde. The 10× photomicrographs were obtained as described previously (21). For the 100× magnification photomicrographs, the stained cells were transferred to micro slides and covered with cover glasses, and photographed by using a Zeiss Atto Arc HBO 110W upright differential interference contrast/fluorescence microscope (Zeiss, Thornwood, NJ). Images were processed by using Adobe Photoshop software.

Flow Cytometric DNA Analysis of NCI-H69 Cell Apoptosis. NCI-H69 cells (2×10^6) were seeded in 25 cm^2 flasks in 5 mL of RPMI 1640 containing 10% FBS in the presence of water or 40 μmol/L naltrindole for the indicated times. Cells were harvested and washed twice with 2 mL of Hanks' balanced saline. The washed cells were fixed in 70% alcohol and washed with PBS and then resuspended in 1 mL of PBS. Cells were stained by 12.5 μg/mL PI for 1 hour before the DNA content of cells was determined by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA). DNA histograms were obtained in an ungated mode. The fraction of the sub-G_0 (apoptotic cells), G_0-G_1, S, and G_2-M cells was calculated by CellQuest (Becton Dickinson), and the sum was normalized to 100%.

Western Blot Analysis of Phosphorylation of Akt/PKB, PDK1, GSK-3, and FKHR. The proteins from cell lysates were separated on10% SDS-PAGE gels and blotted onto nitrocellular membranes according to the manufacturer's protocols (Cell Signaling, Beverly, MA). Phosphorylated Akt/PKB, phosphoinositide-dependent kinase-1 (PDK1), FKHR, and GSK-3 and unphosphorylated Akt/PKB and PDK1 on the membranes were then probed by anti-phosphorylated (anti-p)-Akt/PKB (S-473 and T-308), anti-p-PDK1 (S-241), anti-p-FKHR, anti-p-GSK-3β, anti-Akt/PKB, and anti-PDK1 according to the manufacturer's protocol (New England BioLabs/Cell Signaling), respectively. The phosphorylated and unphosphorylated kinase bands were identified by the Amersham ECL-Western blot analysis kit (Amersham Biosciences, Piscataway, NJ). All anti-p-Akt/PKB antibodies are not isoform-specific; therefore, the detected band should be read as the total phosphorylation at this homologous site. For instance, p-Akt/PKB (S-473) refers to the total p-Akt1/PKBα on serine 473, p-Akt2/PKBβ on serine 474, and p-Akt3/PKBγ on serine 472 throughout the report. Protein concentrations were determined by the *RC DC* protein assay kit (Bio-Rad, Hercules, CA). The absorbance of bands was quantified by a GS-700 Imaging densitometer and its accompanying software, Molecular Analysis (Bio-Rad) or ImageQuant (Molecular Dynamics). The Western blotting images were processed with Adobe Photoshop software.

Naltrindole-Induced Gene Expression in NCI-H69 Cells. The Atlas cDNA Human Apoptosis Arrays (CLONTECH, Palo Alto, CA) were used according to the manufacturer's protocol to detect the changes in gene expression in NCI-H69 cells after cells were treated or untreated with 40 μmol/L naltrindole for 48 hours. The intensity of hybridization signals on a phosphorimaging screen was detected and assessed by Molecular Dynamics Storm 840 Scanner and ImageQuant v1.2 semi-quantitatively (Molecular Dynamics, Sunnyvale, CA). The results were the average of two independent experiments.

RNA Interference. Human embryonic kidney 293 (HEK 293) cells in 24-well or 96-well plates were transfected with Akt1 small interference (si)RNA (siRNA SMARTpool Akt1, Dharmacon, Chicago, IL) following the Lipofectamine 2000 protocol from the manufacturer (Invitrogen). A nonspecific siRNA control pool (Dharmacon) was used as a negative control. Specific reduction of targeted Akt1/PKBα expression level was confirmed by Western blot analysis with anti-Akt1/PKBα monoclonal antibodies (clone SKB1, Upstate Biotechnology, Lake Placid, NY).

Statistical Analysis. Either the one-way ANOVA (Dunnett's method) or Student's *t* test was performed for statistical significance evaluation with the GraphPad Prism program. Statistically significant differences between samples and controls are indicated by one asterisk ($P < 0.05$) and double asterisks ($P < 0.01$).

RESULTS

Inhibition of SCLC Cell Growth by Naltrindole. SCLC cells are neuroendocrine cells (20) expressing all three cloned opioid receptors (22–24). Earlier reports on the apparently inconsistent effects of opioids on cell growth (23, 25) prompted us to survey a series of classical opioid agonists and antagonists in tumor cells. Our preliminary survey of the opioid compounds for the inhibition of NCI-H69 cell growth led to the finding of naltrindole as an inhibitor of NCI-H69 cell growth. We then evaluated the effect of naltrindole on the cell growth of three SCLC cell lines, NCI-H69, NCI-H345, NCI-H510, which have been characterized as the classic SCLC cell lines (20). The inhibition of NCI-H69 cell growth in culture by naltrindole was in a time- and dose-dependent manner (Fig. 1*A*). The IC_{50} values for naltrindole were 25 μmol/L (3 days) and 10 μmol/L (6 days) under the experimental conditions (Fig. 1*A*). Under similar conditions, the universal opioid antagonist naltrexone (1–100 μmol/L) did not inhibit NCI-H69 cell growth (Fig. 1*A*). Naltrindole had a similar dose-response inhibition curve against NCI-H345 cell growth with an $IC_{50} \approx 40$ μmol/L (3 days), whereas naloxone did not inhibit NCI-H345 cell growth (Fig. 1*B*). Inhibition of NCI-H510 cell growth by naltrindole had an $IC_{50} \approx 55$ μmol/L (Fig. 1*C*), which was higher than those of both NCI-H69 and NCI-H345. Furthermore, the clonogenic formation assay showed that naltrindole significantly blocked the colony formation of NCI-H69 cells in soft agar (Fig. 1*D*). At low micromolar concentrations (10 and 20 μmol/L), naltrindole inhibited the colony formation by 39% ($P < 0.01$) and 72% ($P < 0.01$), respectively (Fig. 1*D*). Similar to the observation obtained from the MTT assay, the universal opioid antagonist naloxone had no significant effect on the colony formation under the same conditions (Fig. 1*D*).

A Putative Nonclassic Naltrindole-Binding Site in NCI-H69 Cells. If naltrindole acts via a classic opioid binding site to inhibit SCLC cell growth, other opioid compounds should have some effect on naltrindole-mediated inhibition of cell growth. To address this question, we tested compounds that are either classic opioid agonists or antagonists on naltrindole-mediated cell growth. Opioid agonists morphine, etorphine, DAMGO, and DPDPE, partial agonist/antagonist diprenorphine, and universal antagonists naloxone and naltrexone do not affect the naltrindole-mediated inhibition of cell growth (Fig. 1*E-G*). Our radio-ligand binding experiments also showed that a low affinity naltrindole-binding site existed on the cell membrane preparations in addition to a classic naltrindole-binding site (data not shown). These results together clearly show that naltrindole inhibition of SCLC cell growth does not involve classic opioid receptors, but a nonclassic naltrindole-binding site.

Constitutive Phosphorylation of Akt/PKB in SCLC Cells. It was shown that PI3K activity was constitutively active in a panel of SCLC cell lines and that the PI3K inhibitor LY294002 blocked SCLC cell growth by inhibiting the Akt/PKB activity (26). Thus, we examined whether p-Akt/PKB was constitutive. Three cell lines, NCI-H69, NCI-H345, and NCI-H510, with different PI3K activities (26) were chosen for the examinations. Figure 2 shows that a high level of p-Akt/PKB (S-473)

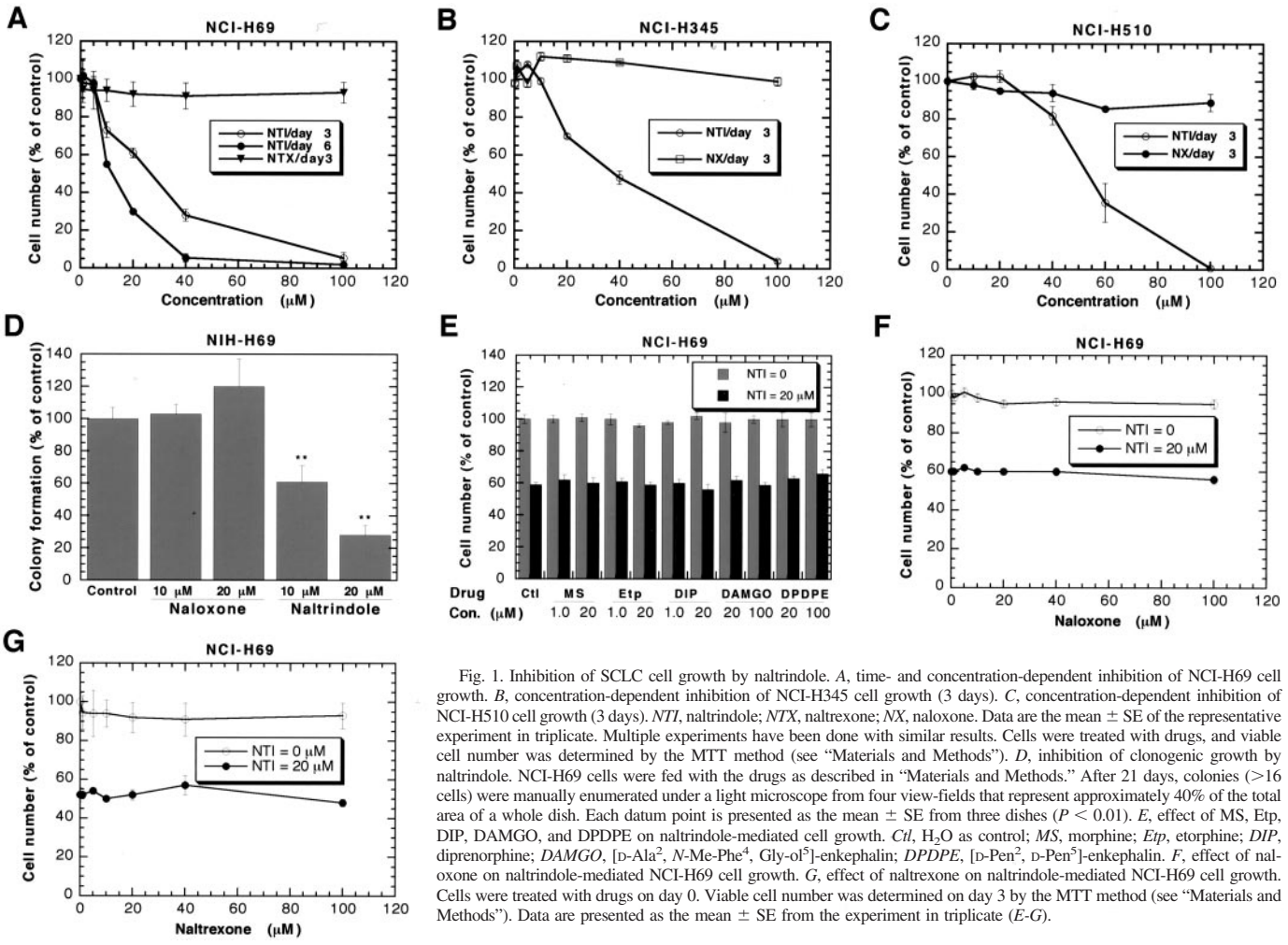

Fig. 1. Inhibition of SCLC cell growth by naltrindole. *A*, time- and concentration-dependent inhibition of NCI-H69 cell growth. *B*, concentration-dependent inhibition of NCI-H345 cell growth (3 days). *C*, concentration-dependent inhibition of NCI-H510 cell growth (3 days). *NTI*, naltrindole; *NTX*, naltrexone; *NX*, naloxone. Data are the mean ± SE of the representative experiment in triplicate. Multiple experiments have been done with similar results. Cells were treated with drugs, and viable cell number was determined by the MTT method (see "Materials and Methods"). *D*, inhibition of clonogenic growth by naltrindole. NCI-H69 cells were fed with the drugs as described in "Materials and Methods." After 21 days, colonies (>16 cells) were manually enumerated under a light microscope from four view-fields that represent approximately 40% of the total area of a whole dish. Each datum point is presented as the mean ± SE from three dishes ($P < 0.01$). *E*, effect of MS, Etp, DIP, DAMGO, and DPDPE on naltrindole-mediated cell growth. *Ctl*, H_2O as control; *MS*, morphine; *Etp*, etorphine; *DIP*, diprenorphine; *DAMGO*, [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]-enkephalin; *DPDPE*, [D-Pen^2, D-Pen^5]-enkephalin. *F*, effect of naloxone on naltrindole-mediated NCI-H69 cell growth. *G*, effect of naltrexone on naltrindole-mediated NCI-H69 cell growth. Cells were treated with drugs on day 0. Viable cell number was determined on day 3 by the MTT method (see "Materials and Methods"). Data are presented as the mean ± SE from the experiment in triplicate (*E-G*).

was observed in NCI-H69 cells, a lower level in NCI-H345 cells, and barely detectable amount in NCI-H510 cells. The p-Akt/PKB (T-308) levels detected by using the commercial anti-p-Akt/PKB (T-308) antibody were very low compared with those of p-Akt/PKB (S-473). [Note: The positive control shows that the anti-Akt/PKB (T-308) antibody was of high quality (data not shown).] The p-Akt/PKB (T-308) levels were not drastically different among the three cell lines as shown in Fig. 2. In the presence or absence of serum, no significant change was observed in the phosphorylation of Akt/PKB on S-473 and T-308. Moreover, the levels of total Akt/PKB protein were similar among the three cell lines although NCI-H345 cell line has 40–60% more Akt/PKB protein than the other two cell lines. Thus, the constitutive phosphorylation of Akt/PKB on S-473 in these cell lines exists in the order of NCI-H69>NCI-H345>NCI-H510, which is consistent with the order of PI3K activity in these cell lines determined previously by others (26). This order of constitutive phosphorylation of Akt/PKB on S-473 (Fig. 2) correlates with the sensitivity of the three cell lines to naltrindole treatment as shown in Fig. 1.

Blocking of Akt/PKB Signaling in SCLC by Naltrindole. First, we evaluated the effect of naltrindole on the phosphorylation of Akt/PKB (S-473) in NCI-H69 cells because NCI-H69 cells have the highest level of constitutive phosphorylation of Akt/PKB (S-473) among the three cell lines. Indeed, naltrindole decreased the phosphorylation of Akt/PKB (S-473; Fig. 3). LY294002, which inhibited NCI-H69 cell growth and induced apoptosis (26), was used as a positive control (Fig. 3). LY294002 at 50 μmol/L inhibited 82% of NCI-H69 cell growth (data not shown) in comparison with 72% by naltrindole at 40 μmol/L for 72 hours of incubation (Fig. 1*A*). Figure 3*A* shows that 24 hours after treatment with 40 μmol/L naltrindole and 50 μmol/L LY294002, the level of p-Akt/PKB (S-473) decreased by 56% ($P < 0.01$) and 77% ($P < 0.01$), respectively. Naloxone as a negative control did not cause a significant decrease in p-Akt/PKB (S-473; $P > 0.05$; Fig. 3*B*). The decrease in p-Akt/PKB (S-473; Fig. 3) and the inhibition of cell growth (Fig. 1) are closely correlated and consistent in this study.

Furthermore, as shown in Fig. 3*C*, naltrindole treatment resulted in the inhibition of Akt/PKB kinase activity and then significantly inhibited the phosphorylation of its downstream substrates FKHR/AFX and GSK-3β, respectively. The minor band above the GSK-3β is the GSK-3α band because of the cross-reactivity of anti-p-GSK-3β antibodies. No substantial change in the Akt/PKB protein levels was observed during drug treatment.

We then hypothesized that the higher constitutive Akt/PKB activity the cells have under serum withdrawal, the more dependent the cell survival and growth will be on the kinase activity and the more sensitive the cells would be to the naltrindole inhibition of Akt/PKB activity. This rationale is consistent with our observation that naltrindole treatment led to the cell death (Fig. 1) in the same order as constitutive activity of Akt/PKB in these three cell lines (Fig. 2). In comparison with each control, naltrindole treatment resulted in a 90% reduction of p-Akt/PKB (S-473) for NCI-H69, 62% for NCI-H345, and 41% for NCI-H510 cells, respectively (Fig. 3*D*), which was in the

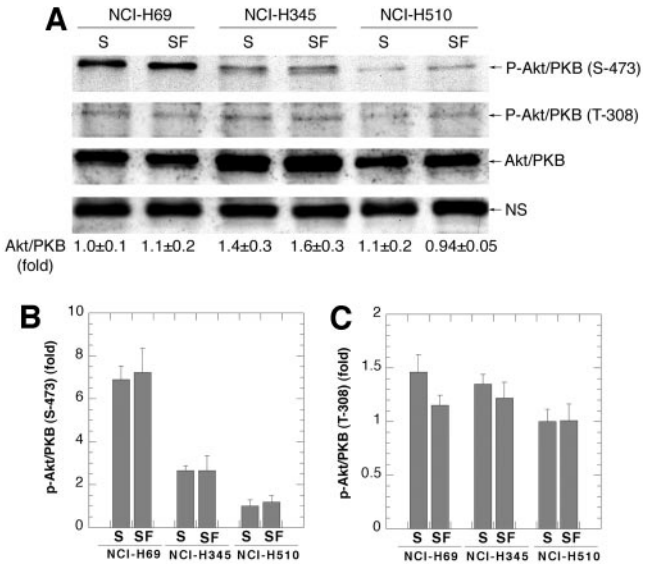

Fig. 2. Constitutive phosphorylation of Akt/PKB in three classic SCLC cell lines. Cells were plated at 2×10^6/well and cultured for 24 hours in the presence or absence of 10% FBS. Cells were then harvested and lysed. Protein samples were analyzed and quantified as described in "Materials and Methods." A nonspecific protein band (NS) of Mr. 33,000 detected by the ECL analysis method was found in all three SCLC cell lines and quantitatively correlated with the amount of total protein loaded, whereas the traditional glyceraldehyde-3-phosphate dehydrogenase band was not shown to be constant among three cell lines. Thus, we used the ratio of the detected protein band to the NS band to correct loading variations among different lanes. Data are presented as the mean ± SE of three independent experiments. In *A*, the relative amount of Akt/PKB in each lane was normalized to that in NCI-H69 cells in 10% serum medium, which was arbitrarily assigned to be 1-fold. In *B* and *C*, the relative amounts of p-Akt/PKB (S-473) and p-Akt/PKB (T-308) were normalized to those in NCI-H510 cells in 10% serum medium. The amounts of p-Akt/PKB (S-473) and p-Akt/PKB (T-308) in NCI-H510 cells were arbitrarily assigned to be 1-fold. *S*, 10% serum medium; *SF*, serum free medium; *NS*, nonspecific protein band.

same order as the sensitivity of cells to naltrindole treatment in cell viability assay (Fig. 1). The reduction of p-Akt/PKB (T-308) by naltrindole seems to be not different among the three cell lines (Fig. 3*D*). Thus, the results suggest that the level of p-Akt/PKB (S-473) in these SCLC cell lines could be an important indicator for the sensitivity to the treatment of Akt/PKB signaling inhibitors.

Finally, we evaluated the effect of naltrindole on phosphoinositide-dependent kinase-1, PDK1, which is an upstream kinase of Akt/PKB (27). Naltrindole reduced the phosphorylation of PDK1 on S-241 by 55%, whereas the PDK1 expression level was unchanged. Under this condition, p-Akt/PKB (S-473) was reduced by 47% whereas the Akt/PKB expression level was stable during drug treatment (Fig. 3*E*). Thus, the reduction of p-Akt/PKB (S-473) is correlated with that of p-PDK1 (S-241).

Naltrindole-Mediated Gene Expression in NCI-H69 Cells. Because naltrindole could inactivate Akt/PKB signaling, resulting in a significant reduction in cell growth by induction of cell death, we surveyed naltrindole-mediated gene expression in NCI-H69 cells with an Atlas cDNA human apoptosis array. Labeled cDNA probes from both untreated and naltrindole-treated NCI-H69 cells for 48 hours were hybridized to an Atlas human apoptosis cNDA array containing 205 different cDNAs. Twelve apoptosis-associated genes have been detected to be up-and down-regulated by >2-fold in response to naltrindole treatment, including *CD27* ligand (*CD70*), Fas-activated serine/threonine kinase (*FAST*), insulin growth factor-like binding protein 2, PCTAIRE protein kinase 1, and UV excision repair protein RAD23 homologue B (Table 1). Up-regulation of proapoptotic genes such as *FAST* and *CD70* further supports the notion that naltrindole induces the apoptosis of NCI-H69 cells by inhibiting the Akt/PKB signaling pathway because both the *Fas* and *CD27* (*CD70* receptor) genes belong to the tumor necrosis factor receptor superfamily, the members of which are important in cell growth and survival (11, 28). Furthermore, Fas is transcriptionally regulated by the transcription factor FKHR (11), which was inactivated after treatment with naltrindole as shown in Fig. 3*C*. These data together provide potential insight into the underlying mechanism of naltrindole-induced apoptosis.

Naltrindole Induces Apoptotic Cell Death. Naltrindole treatment resulted in significant amounts of NCI-H69 cell death (Fig. 4*A*). The population of the cells stained by Hoechst 33342 was much lower in 10 and 100 μmol/L naltrindole-treated cells than in control and 1 μmol/L naltrindole-treated cells (the *bottom panels* of Fig. 4*A*). At 72 hours, most cells underwent a late phase apoptotic cell death in the presence of 100 μmol/L naltrindole. The disintegration of DNAs (10 and 100 μmol/L naltrindole-treated cells, the *bottom panels* of Fig. 4*A*) and intact membranes (the *top panels* of Fig. 4*A*) account for why the majority of cells did not show strong fluorescence in the presence of 10 and 100 μmol/L naltrindole and why the intact cell skeletons were still observed under a phase contrast microscope (Fig. 4*A*). To see the morphological change more clearly, we obtained 100× magnification photomicrographs (Fig. 4*B*). The *arrows* (Fig. 4*B*, *panel d*) point to the individual cells that underwent chromatin condensation and DNA fragmentation, respectively.

To quantify the apoptotic cell death, we further analyzed the DNA content of naltrindole-treated NCI-H69 cells at interval times by the flow cytometric method. As shown in Fig. 4*C*, naltrindole-treated cells underwent apoptotic cell death. Cell counts in sub-G_0 phase (defined as the population of apoptotic cell death) were determined to be 6% for control at 72 h, and 39% and 48% for treatment with 40 μmol/L naltrindole at 48 and 72 h, respectively (Note: Because the controls at 48 and 72 hours had no significant difference in terms of the DNA content analysis, only the control at 72 hours was presented in Fig. 4*C*). The population of control cells at 72 hours in cell cycles was 52% in G_0-G_1, 18% in S, and 23% in G_2-M phase, respectively (the *left panel* of Fig. 4*C*). The population of naltrindole-treated cells at 72 hours in cell cycles was 27% in G_0-G_1, 16% in S, and 8% in G_2-M phase, respectively (the *right panel* of Fig. 4*C*). In comparison with the DNA content of control cells at 72 hours (the *left panel* of Fig. 4*C*), it seems that the apoptotic cell death (sub-G_0, 48%) from the naltrindole-treated cells at 72 hours (the *right panel* of Fig. 4*C*) was mainly contributed by the loss of both G_0-G_1 (from 52% to 27%) and G_2-M phase (from 23% to 8%) cells. The result from the DNA content analysis well correlates with that from the MTT assay. This result is also consistent with that from the PI3K inhibitor LY294002-mediated SCLC cell growth reported by others (26) and correlates with the inactivation of Akt/PKB signaling in NCI-H69 cells (Fig. 3).

Effect of Naltrindole on the Viability of Akt/PKB siRNA-Interfered HEK 293 Cells. Recently, siRNA has been shown to be a powerful mediator to reduce the targeted gene expression in mammalian cells (29). Using this new technology, we evaluated the effect of naltrindole on the viability of Akt/PKB-reduced HEK 293 cells as described in "Materials and Methods." Studies of Akt/PKB-null mice showed that each isoform might also play overlapping roles in cell proliferation and growth (30). Our results aforementioned show that naltrindole targets the Akt/PKB signaling pathway. Figure 5*A* shows that Akt1 siRNA reduced Akt1 and total Akt expression by 65% and 30%, respectively, in comparison with control siRNA. Because HEK 293 cells express both Akt1 and Akt2, we cotransfected cells with both Akt1 and Akt2 siRNA. Reduction of Akt expression by transfection of cells with Akt1/2 siRNAs has no significant effect on HEK cell viability (Fig. 5*B*), suggesting that the remaining Akt/PKB activity is high enough to maintain survival of the cells under the conditions. However, treatment with naltrindole for 12 hours reduced significantly the number of viable cells in Akt siRNA-transfected cells

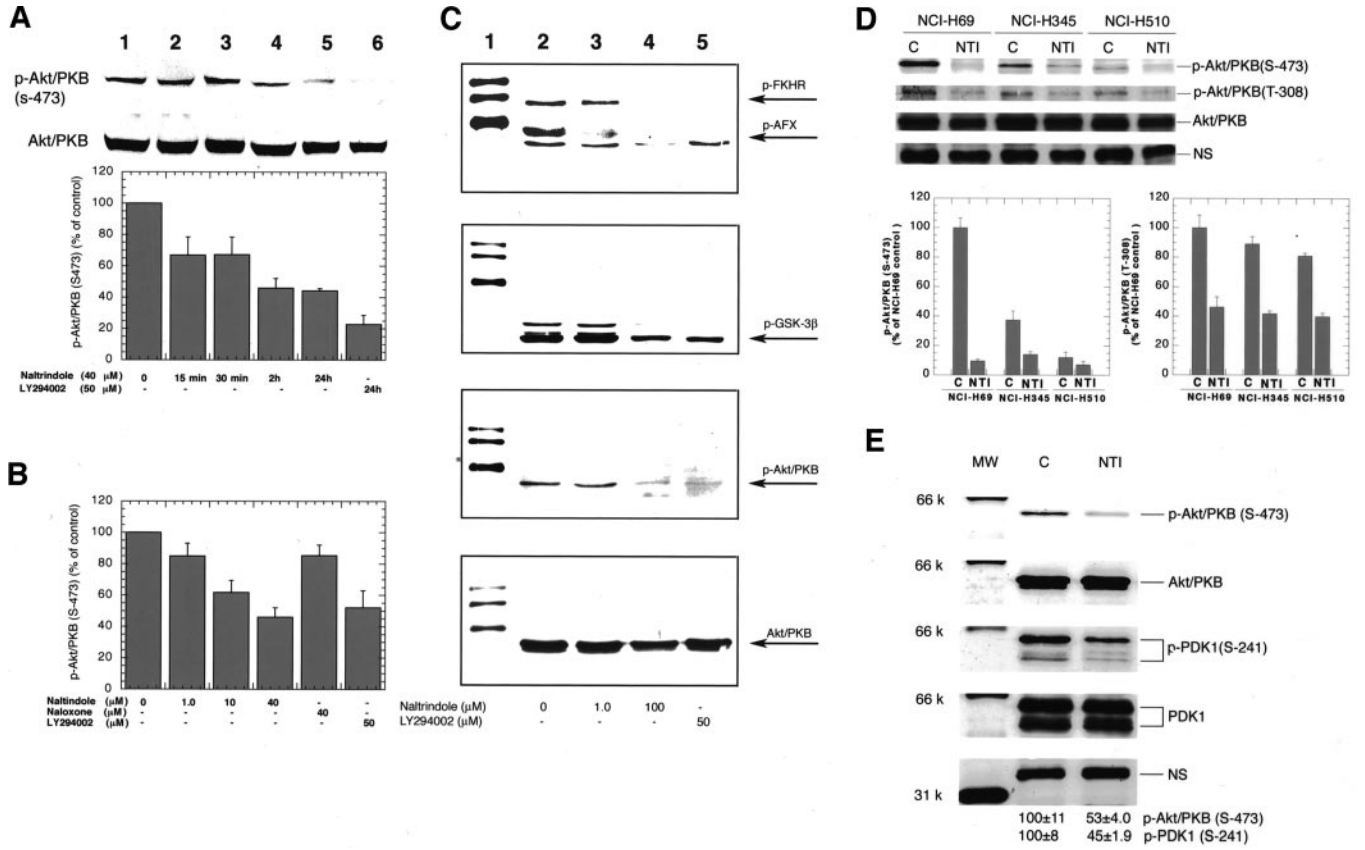

Fig. 3. Inhibition of the Akt/PKB signaling pathway by naltrindole. *A*, time-dependent inhibition of the phosphorylation of Akt/PKB by naltrindole. *Lane 1*, water as control; *Lane 2*, 15 minutes; *Lane 3*, 30 minutes; *Lane 4*, 2 hours; *Lane 5*, 24 hours; 40 μmol/L naltrindole for *Lanes 2–5*; *Lane 6*, treated with 50 μmol/L LY294002 for 24 hours as a positive control. Data are presented as the mean ± SE of at least three independent experiments ($n \geq 3$). The Western blot is one representative of these independent experiments. *B*, concentration-dependent inhibition of the phosphorylation of Akt/PKB by naltrindole. Samples were treated for respective agents for 2 hours. Data are presented as the mean ± SE of at least two independent experiments ($n \geq 2$); *i.e.*, the datum point at 40 μmol/L naltrindole was the average from five independent experiments. *C*, naltrindole blocks the phosphorylation of FKHR, and GSK-3β. *Lane 1*, biotinylated protein markers. NCI-H69 cells were cultured in the presence of 20% FBS and treated with drugs for 30 minutes. The *arrows* point to the corresponding protein bands: Akt/PKB, unphosphorylated Akt/PKB; p-Akt/PKB, phosphorylated Akt/PKB; p-GSK-3β, phosphorylated GSK-3; p-FKHR and p-AFX, phosphorylated FKHR and AFX. *D*, reduction of phosphorylated Akt/PKB (S-473 and T-308) by naltrindole in three classic SCLC cell lines. Single cells (2×10^6) were treated with 100 μmol/L naltrindole for 48 hours as described in "Materials and Methods." The loading variations were corrected as described in the legend to Fig. 2. In the *top panel*, the label *C* refers to the control (untreated). In the *bottom panels*, the relative amount of p-Akt/PKB (S-473) or p-Akt/PKB (T-308) of each sample was normalized to that of untreated NCI-H69 cells, which was assigned to be 100%. For p-Akt/PKB (S-473), data are presented as the mean ± SE of two independent experiments in triplicate. For p-Akt/PKB (T-308), data are presented as the mean ± SE of the representative experiment in triplicate; a similar result was obtained in an additional independent experiment under similar conditions. *E*, effect of naltrindole on PDK1 phosphorylation on S-241. After cells were treated with 40 μmol/L of naltrindole for 2 hours, the cells were harvested and lysed. Protein samples were analyzed and detected as described in "Materials and Methods." Data are presented as the mean ± SE of a representative experiment in three determinations. Similar results were obtained in two additional independent experiments. MW, molecular weight markers.

more than that in control siRNA-transfected cells because naltrindole treatment brought the level of Akt/PKB activity lower than the threshold level of maintaining normal cell survival under these conditions ($P < 0.01$, Fig. 5*B*). Thus, this RNA interference result further confirms that the Akt/PKB signaling pathway is indeed the target of naltrindole treatment.

DISCUSSION

Alkaloid and peptide opioids have been reported to mediate cell growth in a variety of cancer cells (31, 32). The molecular mechanism of this modulation is not clear and controversial. For instance, it has been reported that opioid agonists could either inhibit the growth of

Table 1 *Naltrindole-regulated genes in NCI-H69 cells*

cDNA identified	GenBank accession no.	Upregulation (+)/ downregulation (−) (fold) *	Gene/protein classification †
Ubiquitin C (*UBC*)	M26880	−2.2	Protein turnover
PCTK1	X66363	+2.1	Death kinases
GSTTLp28	U90313	+2.7	Apoptosis-associated proteins
IGF2	M29645	+2.5	Cytokines and chemokines
KIAA0014	D25216	+3.5	Unclassified proteins
IGFBP2	M35410	+2.1	Tumor suppressors
FAST	X86779	+2.3	Death kinases
CD27 LG	L08096	+2.2	Death receptor ligands
hHR23B	D21090	−2.3	DNA damage repair proteins

Abbreviations: *CD27 LG*, *CD27* ligand (also called *CD70*); *FAST*, Fas-activated serine/threonine kinase; *GSTTLp28*, glutathione-*S*-transferase-like protein; *IGF2*, insulin-like growth factor 2; *IGFBP2*, insulin-like growth factor-binding protein 2; *PCTK1*, PCTAIRE protein kinase 1; *hHR23B*, UV excision repair protein RAD23 homolog B.

* Fold differences were determined by calculating the treated:untreated ratio for each gene. An average fold change in each gene was obtained from two independent experiments in duplicate.

† For the sake of simplicity, when the gene belongs to more than one category, only one category is presented here under gene/protein classification.

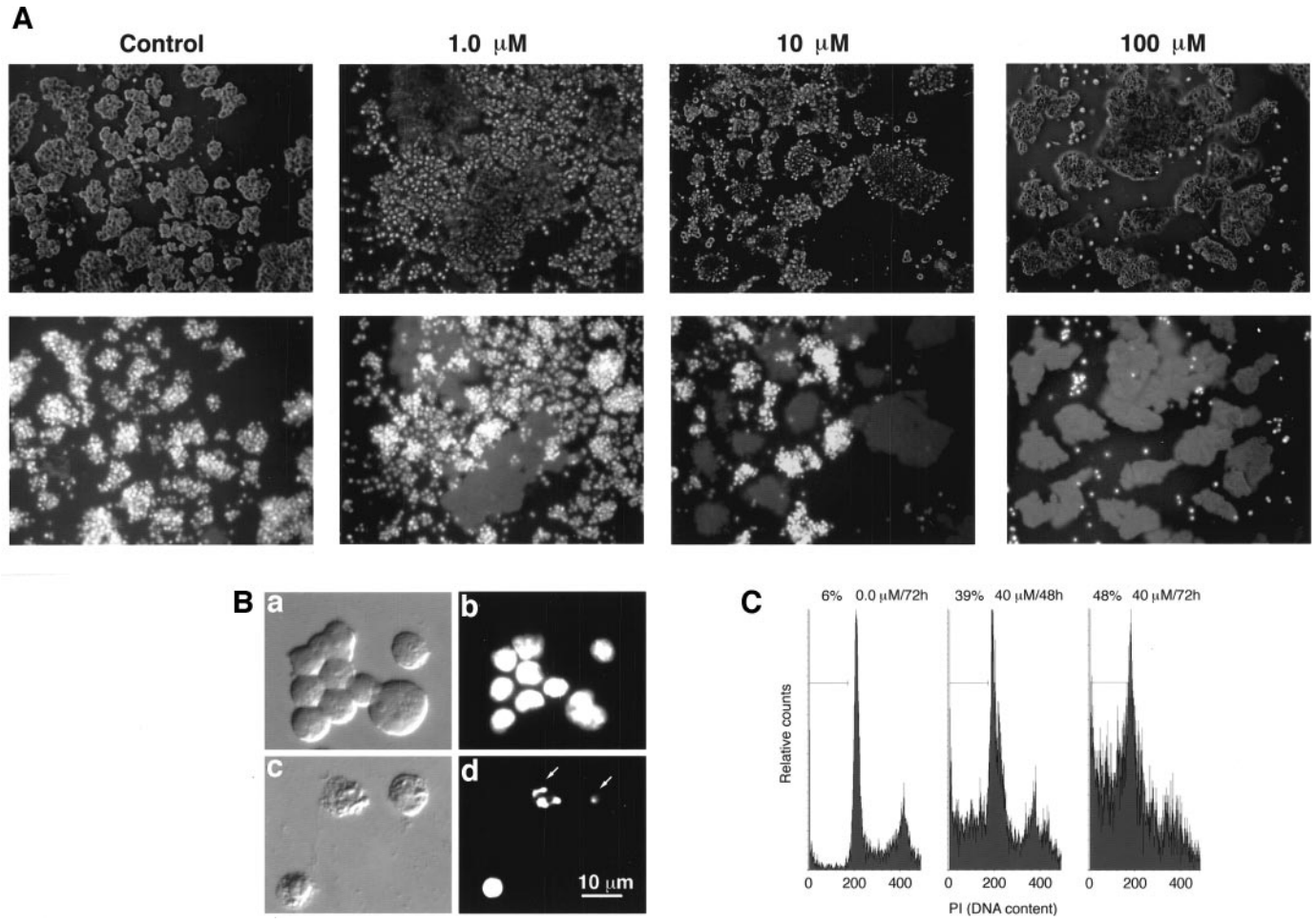

Fig. 4. Apoptosis of NCI-H69 cells induced by naltrindole. *A*, fluorescence analysis of cell death. The *top panels* are the phase contrast photomicrographs of NCI-H69 cells, and the *bottom panels* are the fluorescence photomicrographs. NCI-H69 cells were treated for 72 hours with naltrindole at 0 (control), 1, 10, and 100 μmol/L, respectively. Nuclei were stained with Hoechst 33342. *B*, chromatin condensation and DNA fragmentation. NCI-H69 cells were treated for 72 hours with naltrindole at 40 μmol/L. The *left panels* are differential interference contrast photomicrographs and the *right panels* are fluorescence photomicrographs (100×). *Panels a* and *b*: control cells; *panels c* and *d*: 40 μmol/L naltrindole-treated cells. The *arrows* point to the individual cells that underwent chromatin condensation and DNA fragmentation, respectively. *C*, flow cytometric DNA content analysis. The highest cell count in each panel was normalized to the top scale of the *Y*-axis. The *left panel*, control at 72 hours; the *middle panel*, naltrindole-treated cells at 48 hours; the *right panel*, naltrindole-treated cells at 72 hours. The fraction of the sub-G_0 (apoptotic cells), G_0-G_1, S, and G_2-M cell population was calculated by CellQuest (Becton Dickinson) and the sum was normalized to 100%. A representative DNA content histogram from the triplicate experiments was presented (for experimental details, see "Materials and Methods").

SCLC cells (23) or stimulate the growth of SCLC cells (25). In this study, we show that naltrindole is an inhibitor of the Akt/PKB signaling pathway. Inhibition of Akt/PKB signaling resulted in the inhibition of SCLC cell growth via apoptotic cell death. Other common alkaloid opioid compounds such as naloxone and naltrexone, which usually serve as antagonists for the opioid receptors, did not inhibit the phosphorylation of Akt/PKB and cell growth. Our radioligand binding experiments also showed that a nonopioid naltrindole-binding site exists on cell membrane preparations (data not shown). Together, our results suggest that the nonopioid nature of naltrindole is involved in its newly found function.

Inactivation of Akt/PKB signaling would dephosphorylate a plethora of downstream proapoptotic effectors, including FKHR and GSK-3 (13). The dephosphorylation of these effectors would subsequently initiate apoptotic events (33). The naltrindole inactivation of the Akt/PKB activity resulted in the activation of FKHR and AFX (Fig. 3*C*). Furthermore, naltrindole induces the up-regulation of *CD27* ligand (*CD70*) gene (Table 1). The interaction between CD70 and CD27 could result in apoptosis (34). Moreover, *FAST* was also up-regulated (Table 1), activation of which is required for apoptosis induced by the Fas family proteins (35). The phosphorylation of FKHR-L1 by Akt/PKB generates a binding site for the 14-3-3 family proteins (11). Retaining the complex of FKHR-L1 and 14-3-3 in the cytosol blocks the transcription of genes such as $p27^{kip1}$ and *Fas*, expression of which inhibits cell cycle progression and causes apoptosis (4). Interestingly, PCTAIRE protein kinase 1 has been known to bind 14-3-3 protein (36), suggesting PCTAIRE protein kinase 1 may compete with FKHR for binding to14-3-3, resulting in switching on the transcription of proapoptotic genes such as $p27^{kip1}$ and *CD70*. Thus, up-regulation of *FAST*, PCTAIRE protein kinase 1, and *CD70* together with activation of FKHR by inhibition of Akt/PKB signaling underlines a possible molecular mechanism of naltrindole-induced apoptosis in SCLC cells.

Similar to the activation of FKHR by naltrindole, the activation of GSK-3 by naltrindole could result in the activation of downstream pathways that are repressed by GSK-3 (15), including the phosphorylation of cyclin D, leading to its rapid ubiquitination and proteasomal degradation. Down-regulation of ubiquitin C may also implicate involvement of GSK-3 in naltrindole-induced apoptosis because of the roles of GSK-3 in ubiquitin-mediated degradation of cyclin D (37). Association of cyclin D with CDK2 and CDK4 is required for their kinase activity, which results in the phosphorylation of the RB protein. One of major molecular abnormalities in lung cancer is hyperphosphorylation of the RB protein (3). Our flow cytometric

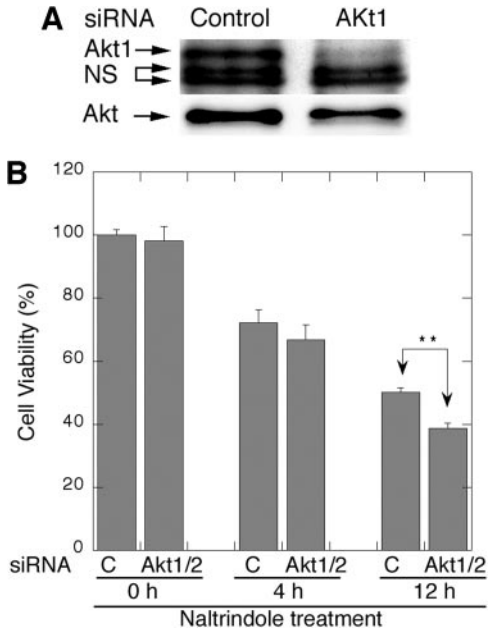

Fig. 5. Effect of naltrindole on cell viability in Akt siRNA-transfected HEK 293 cells. *A*, specific reduction of Akt1 expression level by Akt1 siRNA. HEK 293 cells were transfected with 25 nmol/L Akt1 siRNA or control siRNA for 72 hours as described in "Materials and Methods." Akt1 and total Akt expression levels were analyzed by Western blot analysis with Akt1/PKBα monoclonal antibody and anti-Akt antibody, respectively. Figure 5*A* shows the result of Western blot analysis of the representative experiment. Similar results were obtained in three additional experiments (for 48 and 72 hours). *B*, effect of naltrindole on the viability of Akt1/Akt2 siRNA-transfected cells. After transfection with 20 nmol/L Akt1 and Akt2 together or 40 nmol/L nonspecific siRNA control for 48 hours, cells were treated with 100 μmol/L naltrindole at the indicated times. Cell viability was assayed by MTT as described in "Materials and Methods." Data are presented as the mean $\pm$ SE of the triplicate experiments ($^{**}P < 0.01$). Similar results were obtained in another independent experiment. *C*, nonspecific control siRNA pool; *Akt1/2*, Akt1 and Akt2 siRNA pool.

analysis experiments show that naltrindole significantly reduced the cell population of G_0-G_1 phase and increased the apoptotic population of sub-G_0 phase (Fig. 4*C*). It seems that the inhibition of Akt/PKB-GSK-3β signaling by naltrindole could result in the inhibition of cyclin D/CDK4 activity, leading to the dephosphorylation of the RB protein, and then to arresting the cell cycle progression, and eventually to the apoptosis of SCLC cells.

In this study we also found that the levels of p-Akt/PKB (S-473) correlate with those of PI3K activity (26) among the three tested cell lines. The order of Akt/PKB activity in the three cell lines (Fig. 2) correlates with that of the IC_{50} values of naltrindole against the cell growth of these lines (Fig. 1). Furthermore, the Akt/PKB expression level (Fig. 2) does not correlate with the sensitivity of the tested cell lines to naltrindole treatment (Fig. 1). Together, this suggests that the higher constitutive Akt/PKB activity the Akt/PKB-dependent SCLC cells have, the more sensitive the SCLC cells are to the perturbation of Akt/PKB activity. This result is reminiscent of the observation reported previously for PI3K inhibitor LY294002-induced apoptosis in NSCLC cells, in which the sensitivity of NSCLC cells to LY294002 correlates with the levels of Akt/PKB activity (7). Interestingly, our results also parallel those from the recently reported clinical studies of gefitinib, an inhibitor against epidermal growth factor receptor activity and an anti-NSCLC drug (38, 39). In their studies, only NSCLC cell lines with high constitutive epidermal growth factor receptor activity caused by somatic mutations, which leads to high constitutive Akt/PKB activity, were sensitive to gefitinib treatment. Further work is needed to determine whether the differential sensitivity to naltrindole in SCLC cell lines reflects specific mutations that may occur in the putative naltrindole target (see below).

We have identified naltrindole as an inhibitor of the Akt/PKB signaling pathway and our RNA interference experiments also confirm that naltrindole indeed targets the Akt/PKB signaling pathway, but the direct physiologic target molecule of naltrindole is yet to be identified. On the basis of our current observations aforementioned, we postulate that the direct target of naltrindole may be situated on cell surface. Although the Akt/PKB upstream molecules such as PI3K, PDK1, and PDK2 are possible candidates, other likely candidates would be among those receptors on cell surfaces that are directly involved in controlling SCLC cell growth (40), including the neuropeptide receptors (ref. 41 and refs. 2–5 cited therein) and epidermal growth factor receptor, platelet-derived growth factor receptor, c-Kit, and c-Met, all of which are receptor tyrosine kinases (40). Because the IC_{50} values of naltrindole to inhibit the SCLC cell growth were at low micromolar ranges (10–55 μmol/L), it was still possible that other targets besides Akt/PKB signaling might be affected in part to render the modulation of cell growth, and we did not investigate this aspect. Finally, another possibility of naltrindole inhibition could be in part caused by the physical interaction with lipid membranes of the cell. Because the naltrindole's parental compound naloxone and other alkaloid opioids with similar structures did not have such effect on cell growth and Akt/PKB activity and because naltrindole and naloxone are indeed quite water-soluble, such nonspecific lipid effect could be minimum under the experimental conditions. However, it is yet to be determined whether the naltrindole inhibition of Akt/PKB signaling is attributable to specific lipid interaction with its target molecule on cell membranes. Thus, one of our ongoing efforts is to identify and characterize the putative naltrindole-binding protein (binding site) that is responsible for the naltrindole-mediated apoptosis in SCLC cells. It will be more feasible to further characterize the putative binding site with a higher affinity compound and finally to clone and purify the site. Such work will definitely provide more insights into the mechanism of action of naltrindole in SCLC cells. Nonetheless, using naltrindole as a lead, we should now have a starting point to design, synthesize, and screen more potent and selective compounds that inhibit the Akt/PKB signaling pathway.

Cancer-induced pain is experienced by 30 to 50% of all cancer patients and 75 to 90% of advanced cancer patients, respectively (42). Treatment of cancer pain is a major area where opioid drugs are clinically used (43), and the development of tolerance and physical dependence is a major side effect in using these opioid drugs (43). Hence, it is both pharmacologically and clinically important that there is a way to maximize the relief of pain and to minimize the side effects. Recently, Zhu *et al.* (44) showed that the δ-opioid receptor knockout mice retained the supraspinal δ-like analgesia and diminished morphine tolerance. Naltrindole has been shown to be a potent agent for blocking the chronic morphine-induced tolerance and physical dependence in animals (45). Thus, the results from others and us together indicate that naltrindole and its derivatives may be used as a lead to develop a new type of agent that could act both to minimize the chronic morphine-like drug side effects and to be an anticancer drug candidate at the same time.

In summary, we have found naltrindole to be a new inhibitor of the Akt/PKB signaling pathway. Naltrindole inhibits the constitutively active Akt/PKB signaling-dependent survival and growth of three tested SCLC cell lines. We found that among these three cell lines the higher level of constitutive phosphorylation of Akt/PKB (S-473) the cells have, the more sensitive the cells are to the naltrindole inhibition of Akt/PKB signaling. Blocking of the upstream PDK1 kinase is concurrent with the inhibition of Akt/PKB activity, which leads to the activation of two major proapoptotic signaling pathways, FHKR and GSK-3β, resulting in the up-regulation of other proapoptotic gene products such as *PCTK1*, *FAST*, *CD70*, and ubiquitin C. Together, our

findings provide a mechanistic explanation for the anticancer properties of naltrindole. Additional work is required to elucidate the detailed mechanism of action of naltrindole and to clone, purify, and fully characterize the upstream target molecule (or binding site) in SCLC cells.

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