The phosphatidylinositol-3-kinase inhibitor PX-866 overcomes resistance to the epidermal growth factor receptor inhibitor gefitinib in A-549 human non–small cell lung cancer xenografts

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Abstract

Epidermal growth factor receptor (EGFR) inhibitors such as gefitinib show antitumor activity in a subset of non – small cell lung cancer (NSCLC) patients having mutated EGFR. Recent work shows that phosphatidylinositol-3-kinase (PI3-K) is coupled to the EGFR only in NSCLC cell lines expressing ErbB-3 and that EGFR inhibitors do not inhibit PI3-K signaling in these cells. The central role PI3-K plays in cell survival suggests that a PI3-K inhibitor offers a strategy to increase the antitumor activity of EGFR inhibitors in resistant NSCL tumors that do not express ErbB-3. We show that PX-866, a PI3-K inhibitor with selectivity for p110α, potentiates the antitumor activity of gefitinib against even large A-549 NSCL xenografts giving complete tumor growth control in the early stages of treatment. A-549 xenograft phospho-Akt was inhibited by PX-866 but not by gefitinib. A major toxicity of PX-866 administration was hyperglycemia with decreased glucose tolerance, which was reversed upon cessation of treatment. The decreased glucose tolerance caused by PX-866 was insensitive to the AMP-activated protein kinase inhibitor metformin but reversed by insulin and by the peroxisome proliferator-activated receptor-γ activator pioglitazone. Prolonged PX-866 administration also caused increased neutrophil counts. Thus, PX-866, by inhibiting PI3-K signaling, may have clinical use in increasing the response to EGFR inhibitors such as gefitinib in patients with NSCLC and possibly in other cancers who do not respond to EGFR inhibition. [Mol Cancer Ther 2005;4(9):1349 – 57]

Introduction

Increased cell survival is a fundamental characteristic of cancer cells and limits the effectiveness of cancer therapy (1). An important mechanism for increased cell survival in many cancers is mediated by the phosphatidylinositol-3-kinase (PI3-K)/Akt (protein kinase B) signaling pathway that is activated by receptor and oncogenic protein tyrosine kinases (2). Eight mammalian PI3-Ks are divided into three main classes: class I PI3-Ks phosphorylate membrane phosphatidylinositol to give $PI(3, 4, 5)P_3$ that recruits the cytoplasmic serine/threonine kinase Akt by binding to its pleckstrin homology domain. Membrane-associated Akt is activated by Ser^{473} phosphorylation by membrane-associated phosphoinositide-dependent kinase-1 (3) and Thr^{308} phosphorylation by a second incompletely characterized phosphoinositide-dependent kinase-2 (4). Activated Akt detaches from the plasma membrane and moves to the cytoplasm and the nucleus, where it phosphorylates a battery of targets to prevent the expression of death genes, and induces cell survival (5). PI3-K activity is increased in human small cell lung cancer, ovarian, head and neck, urinary tract, colon, and cervical cancers (6–8). The tumor suppressor protein PTEN (phosphatase and tensin homologue deleted on chromosome 10), a dual specificity tyrosine-threonine/PI-3 phosphatase, prevents the accumulation of $PI(3,4,5)P_3$ and attenuates PI3-K signaling (9). PTEN is mutated or deleted in a variety of human cancers including advanced prostate, endometrial, renal, glial, melanoma, and small cell lung cancers (10).

The protein kinase family has >800 human members (11) among which receptor protein tyrosine kinases are frequently targets for cancer therapy. They include the epidermal growth factor receptor (EGFR, ErbB-1, HER1), that when activated by ligand binding to its extracellular domain, homodimerizes or heterodimerizes with any of three other family members, ErbB-2 (HER2), ErbB-3 (HER3), and ErbB-4 (HER4), leading to autophosphorylation of cytoplasmic COOH-terminal tyrosine residues. These phosphorylations recruit signal transducers leading to activation of signaling pathways that include the

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Ras/mitogen-activated protein kinase kinase/mitogen-activated protein kinase pathway, the signal transducers and activators of transcription pathway, and the PI3-K/Akt survival pathway. EGFR is amplified or overexpressed in a wide range of human cancers where it is thought to play an important role in tumor progression (12). In non–small cell lung cancer (NSCLC), EGFR expression is correlated with decreased patient survival (13). A number of small molecule inhibitors of the EGFR kinase as well as EGFR monoclonal antibodies are under development or approved for clinical use. Gefitinib (ZD 1839, Iressa) is a small-molecule EGFR inhibitor that when given to patients with relapsed NSCLC has shown a response rate of 10% to 20% and stabilized the disease in another 20% to 30% of patients (14). However, the addition of gefitinib to chemotherapy in untreated patients with NSCLC had no effect on overall survival, time to progression, or response rate (15). A majority, but not all, NSCLC patients responding to single-agent gefitinib contain somatic mutations of unknown functional significance in the EGFR tyrosine kinase domain (16). However, there are also NSCLC patients who do not have mutated EGFR receptors and who may derive benefit from gefitinib and other EGFR inhibitors. Furthermore, although activating mutations of the EGFR are rare in human colorectal cancer and glioblastoma (17), some of these tumors may be responsive to EGFR inhibitors (18). A recent study has shown that gefitinib inhibits cell growth and down-regulates PI3-K signaling only in NSCLC cell lines with ErbB-3 expression (19). This is because PI3-K couples to ErbB-3 leading to PI3-K/Akt signaling activation only in NSCLC cell lines with either wild-type or mutant EGFR receptor and ErbB-3. Gefitinib is able to block the association of PI3-K with ErbB-3 thus preventing PI3-K/Akt activation in these cell lines. The central role PI3-K plays in determining the response to gefitinib suggests that an inhibitor of PI3-K may provide a strategy to increase the antitumor activity of gefitinib in resistant NSCLC tumors that do not express ErbB-3. PX-866 is a novel inhibitor of PI3-K that is currently in advanced preclinical development as an antitumor agent (20). We used the A-549 human NSCLC cell line with mutant active N-Ras that does not express ErbB-3 and is resistant to gefitinib (19). We found that in A-549 tumor xenografts gefitinib did not inhibit PI3-K/Akt signaling and the administration of PX-866 either i.v. or orally markedly potentiated the antitumor activity of gefitinib. We also report on the toxicity of long-term administration of PX-866, showing that it increases blood glucose associated with a decrease in insulin sensitivity.

Materials and Methods

Compounds

PX-866 [acetic acid (1*S*,4*E*,10*R*,11*R*,13*S*,14*R*)-4-diallylaminomethylene-6-hydroxy-1-methoxymethyl-10,13-dimethyl-3,7,17-trioxo-1,3,4,7,10,11,12,13,14,15,16,17-dodecahydro-2-oxa-cyclopenta[a]phenanthren-11-yl ester] was synthesized as previously described (21). For i.v. administration to mice, PX-866 was dissolved at 10 mg/mL in 5% ethanol in 0.9% NaCl and for oral administration at 5 mg/mL in 5% ethanol in water. Gefitinib was obtained from AstraZeneca (Macclesfield, United Kingdom) and suspended at 7.5 mg/mL in 0.1% Tween 20 in water for oral administration. Rabbit-purified anti-phospho-Ser^{473}-Akt antibody, anti-Akt antibody, anti-phospho-Tyr^{1086}-EGF-receptor antibody, and anti-EGFR antibody were obtained from Cell Signaling Technology (Beverly, MA). Human recombinant p110α/p85α, p110β/p85α, p120γ, and p110δ/p85α PI3-Ks were obtained from Upstate (Charlottesville, VA). Metformin hydrochloride was obtained from Spectrum Chemical (Gardena, CA); pioglitazone hydrochloride and recombinant human insulin from Sigma Chemical Co. (St. Louis, MO).

Cells

A-549 NSCLC cells were obtained from the American Tissue Type Collection (Rockville, MD). The cells were grown in humidified 95% air, 5% CO_2 at 37°C in DMEM supplemented with 10% fetal bovine serum. All cell lines were tested to be *Mycoplasma* free using a PCR ELISA kit (Roche Diagnostics, Inc., Indianapolis, IN).

Measurement of PI3-K

The ability of PX-866 to inhibit recombinant human p110α/p85α, p110β/p85α, p120γ, and p110δ/p85α was measured by the [^{32}P]γ-ATP-dependent phosphorylation of phosphatidylinositol as described by Stirdivant et al. (22). Inhibition of cellular PI3-K was measured as the ratio of phospho-Ser^{473}-Akt to total Akt measured by Western blotting, as previously described (20).

Antitumor Studies

Approximately 10^7 A-549 NSCLC cells in log cell growth were injected s.c. in 0.2 mL PBS into the flanks of severe combined immunodeficient (SCID) mice. When the tumors reached 100 or 600 mm^3, the mice were stratified into groups of eight animals having approximately equal mean tumor volumes and drug administration was started. Dosing was every other day with gefitinib at 75 mg/kg orally; PX-866 at 4, 9, or 12 mg/kg i.v.; PX-866 at 1, 2.5, and 3 mg/kg orally; or PX-866 given 4 hours before gefitinib. Animals were weighed weekly and tumor diameters were measured twice weekly at right angles (d_{short} and d_{long}) with electronic calipers and tumor volumes calculated by the formula volume = $(d_{short})^2 \times (d_{long}) / 2$ (23). When the tumor reached ≥2,000 mm^3 or became necrotic, the animals were euthanized.

Pharmacodynamic Studies

A-549 NSCLC cells (10^7) were injected s.c. into the flanks of male SCID mice and allowed to grow to ~300 mm^3. Mice were given PX-866 12 mg/kg i.v., 3 mg/kg orally, and gefitinib 75g/kg orally, every other day for 5 days. Tumors were removed 24 hours after the last dose and immediately frozen in liquid N_2. For assay, the tumors were homogenized in 50 mmol/L HEPES buffer (pH 7.5), 50 mmol/L NaCl, 1% NP40, and 0.25% sodium deoxycholate and Western blotting done using anti-phospho-Ser^{473}-Akt and anti-Akt antibodies. Tumor Akt activity was expressed as the ratio of phospho-Ser^{473}-Akt to total Akt.

Toxicity Studies

Male SCID mice were given PX-866 at 10 mg/kg i.v., or 3 and 1.5 mg/kg orally, every other day for 14 doses. C57Bl/6 mice were given PX-866 at 3 mg/kg orally every other day for 15 doses. The mice were killed 24 hours after the last dose and changes in body weight, blood lymphocyte, neutrophil, RBC, platelet counts, serum glucose, aspartate aminotransferase, and alanine aminotransferase were measured.

Glucose Tolerance Studies

Female C57B1/6 mice were fasted overnight and given a single dose of D(+)-glucose (1 mg/kg) as a 0.1 g/mL solution orally. Blood was collected at 0, 10, 20, 30, 60, 90, 120, and 180 minutes and plasma glucose measured using a blood glucose kit (Sigma Chemical) to obtain a plasma glucose area under the curve ($AUC_{0\text{-}180\ minutes}$). Mice were given PX-866 10 mg/kg orally as a single dose and glucose given 4 hours later, or 3 mg/kg PX-866 orally every other day for 20 doses and glucose given 24 hours and 8 days after the last dose. Metformin was given at 250 mg/kg orally daily for 5 days (24) and 10 mg/kg pioglitazone i.p. daily for 7 days (25) before the glucose administration. Human recombinant insulin was given at 0.075 μg/kg i.p. (26) at the same time as glucose administration.

Bone Marrow Colony Formation

After sacrifice, mouse bone marrow was extracted from each femur and RBC lysed with 0.2% hypotonic NaCl followed by the addition of a 1.6% hypertonic NaCl. Approximately 20,000 cells were plated in 1 mL of Methocult GF M3434 (Stemcell Technologies, Inc., Vancouver, British Columbia, Canada) containing 1% methylcellulose in Iscove's minimum essential medium, 15% fetal bovine serum, 1% bovine serum albumin, 10 μg/mL recombinant human insulin, 200 μg/mL human transferrin, 10 mmol/L β-mercaptoethanol, 2 mmol/L L-glutamine, 50 ng/mL recombinant mouse stem cell factor, 10 ng/mL recombinant mouse interleukin-3, 10 ng/mL recombinant human interleukin-6, and 3 units/mL recombinant erythropoietin. Cells were plated in triplicate and grown at 37°C and 5% CO_2 in a humid environment for 14 days before scoring. Colonies (>40 cells per colony) or clusters (3–40 cells) were scored and growth of colony-forming unit granulocyte, erythroid, macrophage, megakaryocyte; burst-forming units erythroid; and CFU granulocyte macrophage, assessed using standard criteria (27). Qualitative observations were made on background levels of single cells.

Table 1. Inhibition of PI3-Ks by PX-866 and wortmannin

PI3-K	PX-866 IC_{50} (nm)	Wortmannin IC_{50} (nm)
p110α/p85α	5.5	4.0
p110β/p85α	>300	0.7
p120γ	9.0	9.0
p110δ/p85α	2.7	4.1

NOTE: Recombinant PI3-Ks were assayed for activity as described in the text.

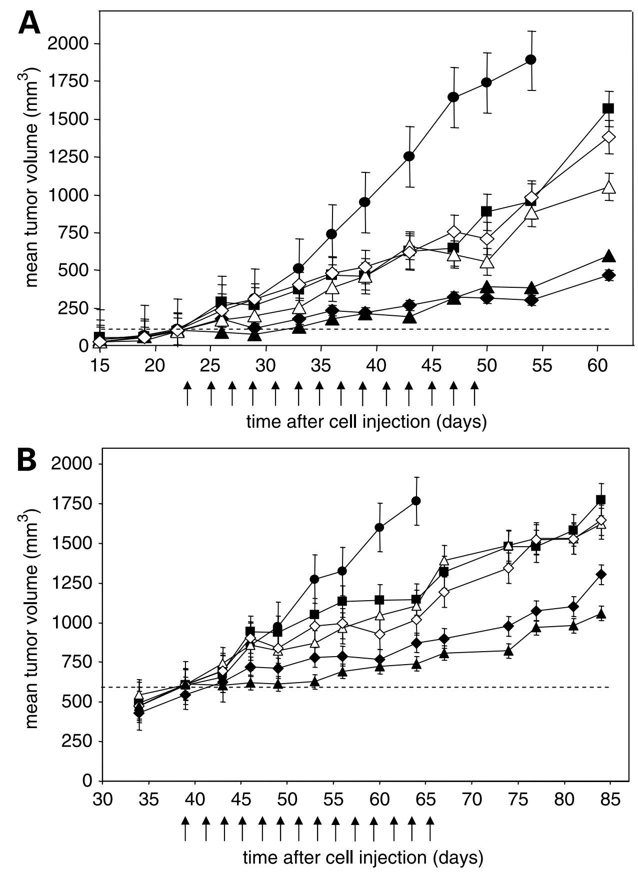

Figure 1. Potentiation of the antitumor activity of gefitinib by PX-866. Female SCID mice were implanted s.c. with 10^7 A-549 human NSCLC cells. **A,** tumors were 100 mm^3 on day 22 (*dashed line*) when dosing was begun every other day for 15 doses (*arrows*) with: ●, vehicle alone; ■, gefitinib (75 mg/kg orally); △, PX-866 (9 mg/kg i.v.); ◇, PX-866 (2.5 mg/kg orally); ▲, PX-866 (9 mg/kg i.v.) 4 h before gefitinib (75 mg/kg orally); and ♦, PX-866 (2.5 mg/kg orally) 4 h before gefitinib (75 mg/kg orally). *Points,* means of eight mice per group; *bars,* SE. **B,** tumors were 600 mm^3 on day 39 (*dashed line*) when dosing was begun every other day for 14 doses (*arrows*) with: ● vehicle alone; ■, gefitinib (75 mg/kg orally); △, PX-866 (12 mg/kg i.v.); ◇, PX-866 (4 mg/kg orally); ▲, PX-866 (12 mg/kg i.v.) 4 h before gefitinib (75 mg/kg orally); and ♦, PX-866 (4 mg/kg orally) 4 h before gefitinib (75 mg/kg orally). *Points,* means of eight mice per group; *bars,* SE.

Results

PI3-K Inhibition

The ability of PX-866 to inhibit recombinant PI3-Ks compared with inhibition by wortmannin is shown in Table 1. PX-866 and wortmannin are potent inhibitors of p110α, p120γ, and p110δ but unlike wortmannin PX-866 is a poor inhibitor of p110β.

Cell Culture Studies

PX-866 inhibited phospho-Akt in A-549 human breast cancer cells in medium containing 10% fetal bovine serum with an IC_{50} of 25 nmol/L. Gefitinib only inhibited phospho-Akt in cells that were serum starved for 24 hours and stimulated with EGF 25 ng/mL but not in medium with 10% fetal bovine serum. This suggests that the PI3-K pathway is stimulated by growth factors in serum, in addition to EGF. Cell growth inhibition studies confirmed previous reports (19) that A-549 cells are resistant to growth inhibition by gefitinib, with an IC_{50} of 1.1 μmol/L. PX-866 at concentrations up to 100 nmol/L did not enhance the growth inhibition by gefitinib.

In vivo Antitumor Studies

Administration of gefitinib at 75 mg/kg orally every other day to mice with 100 mm^3 A-549 human NSCLC xenografts inhibited xenograft growth with a treated versus control of 51 % at the end of the dosing period (Fig. 1A). We have previously reported that PX-866 is approximately four times more potent as an antitumor agent when given orally than given i.v., and doses were adjusted accordingly (Table 2). When given alone to mice with 100 mm^3 A-549 tumor xenografts, PX-866 inhibited tumor growth with treated versus control values of 31% at 9 mg/kg i.v. and 41% at 2.5 mg/kg orally. Preliminary studies showed that PX-866 in combination with gefitinib on an alternating day schedule was more active when given 4 hours before rather than 24 hours after gefitinib (data not shown). When PX-866 was given 4 hours before gefitinib, the combination gave treated versus control values of 22% at 9 mg/kg PX-866 i.v. and 18% at 2.5 mg/kg PX-866 orally. Tumor growth was held stationary for the first half of the treatment period with PX-866 and then began to slowly increase towards the end of the period (Fig. 1A). Increased combination antitumor activity was also seen with very large 600 mm^3 A-549 tumor xenografts (Fig. 1B).

Inhibition of Tumor EGFR and PI3-K Signaling

Administration of gefitnib 75 mg/kg orally to mice with A-549 tumor xenografts every other day for 5 days inhibited tumor phospho-EGFR by 43% but had no significant effect upon tumor phospho-Akt (Fig. 2). PX-866 12 mg/kg i.v. or 3 mg/kg orally, every other day for 5 days, had no significant effect upon tumor phospho-EGFR but inhibited tumor phospho-Akt by 51% and 48%, respectively. The combination of gefitinib and PX-866 inhibited both tumor phospho-EGFR and tumor phospho-Akt. Similar effects were seen in a second study (data not shown). Thus, in A-549 tumor xenografts, the EGFR and PI3-K pathways seem to function independently and to be selectively inhibited by gefitinib and PX-866, respectively.

Toxicity of Long-term PX-866 Administration

The toxicity of long-term administration of PX-866 to SCID mice is summarized in Table 3. There was a decreased gain in body weight over the 4 weeks of treatment with PX-866 at 10 mg/kg i.v. and 3 mg/kg orally to 83% and 28% of the control weight gain, respectively ($P < 0.05$). There was no change in the plasma liver enzymes alanine aminotransferase and aspartate aminotransferase but a significant increase in plasma glucose caused by PX-866 at 1.5 and 3 mg/kg orally of 113% and 142%, respectively ($P < 0.05$). The increase in blood glucose with PX-866 at 10 mg/kg i.v. was 62% but was not significant ($P > 0.05$). There was a significant increase in WBC counts following oral administration of PX-866 due primarily to increased blood neutrophil counts. All of the changes in body weight, plasma glucose, and blood cell counts had returned to normal by 9 days after treatment stopped. The decrease in body weight and an increase in blood glucose were confirmed in two additional studies using SCID mice, but the increase in blood cell counts was less pronounced in these studies (data not shown).

PX-866 and Glucose Tolerance

To gain further insight into the mechanism for the increase in plasma glucose by PX-866, studies were conducted on insulin levels and on glucose tolerance following an oral dose of 1 g glucose/kg to fasted C57Bl/6 mice (Fig. 3). Administration of PX-866 as a single dose of 10 mg/kg orally caused an increase in plasma insulin levels for up to 5 hours. PX-866 also decreased glucose tolerance in the mice leading to an increase in plasma glucose, particularly at time points after 1 hour after glucose administration where plasma glucose was decreasing in nontreated mice but increasing in the PX-866-treated mice. The results expressed as $AUC_{0\text{-}180\ \text{minutes}}$ for all the glucose tolerance studies are shown in Table 4. Treatment with insulin at high doses overcame the increase in plasma glucose caused by PX-866 and significantly decreased the glucose $AUC_{0\text{-}180\ \text{minutes}}$ in both control and PX-866-treated mice. The antihyperglycemic drug metformin had no effect upon the increase in blood glucose by PX-866, but the hypoglycemic thiazolidinedione drug pioglitazone almost completely blocked the increase (Fig. 3; Table 4). Long-term treatment with PX-866 at 9 mg/kg i.v. every other

Table 2. Antitumor activity of PX-866 in combination with gefitinib

Treatment and route	Dose (mg/kg)	Schedule	Tumor T/C %	PX-866 4 h before gefitinib, tumor T/C %
Gefitinib orally	75	QOD × 14	50.8	—
PX-866 i.v.	4	QOD × 14	65.3	20.5
PX-866 i.v.	9	QOD × 14	31.5	22.3
PX-866 orally	1	QOD × 14	54.8	40.8
PX-866 orally	2.5	QOD × 14	40.8	18.1

NOTE: Female SCID mice were implanted s.c. in the flank with 10^7 A-549 human NSCLC cells. Tumors were allowed to grow to a mean volume of 100 mm^3 before drug treatment was started every other day for 14 doses. Antitumor activity is expressed as the % volume of the treated tumor T/C % at the end of the dosing period. There were eight mice in each group and all differences are $P < 0.01$.
Abbreviation: T/C, tumor/control.

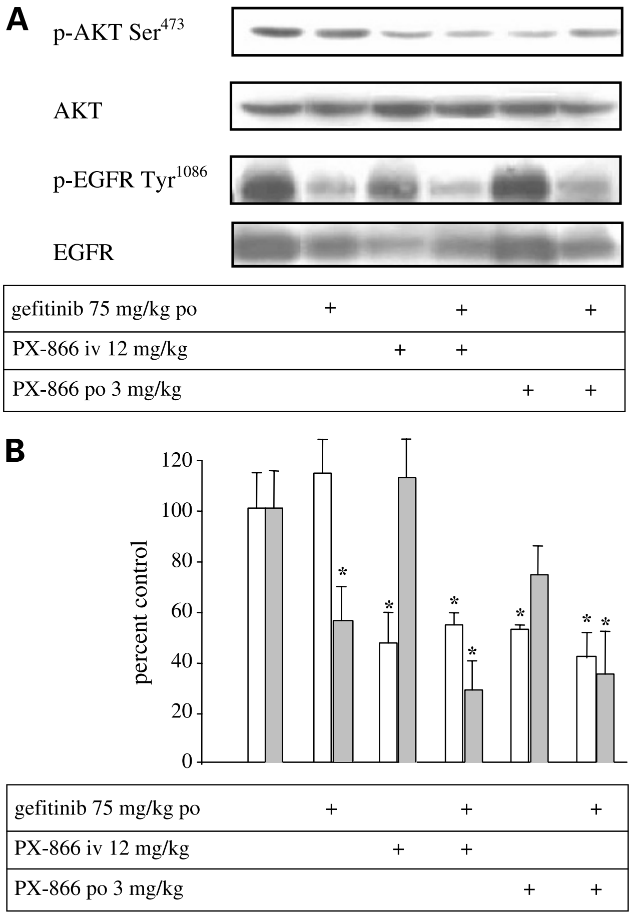

Figure 2. Inhibition of EGFR and phospho-Akt in A-549 NSCLC xenografts by gefitinib and PX-866. Female SCID mice with 300 mm^3 A-549 NSCLC xenografts were given gefitinib (75 mg/kg orally), PX-866 (12 mg/kg i.v.), or PX-866 (3 mg/kg orally), alone or with the PX-866 4 h before the gefitinib, daily every other day for 5 d. Twenty-four hours after the last dose, tumors were removed for measurement of phospho-Akt/total Akt (*open columns*) or phospho-EGFR (*shaded columns*). **A,** typical Western blots showing phospho-Akt, Akt, phospho-EGFR, and EGFR. **B,** *columns,* mean values of three mice; *bars,* SE. *, $P < 0.05$ compared with nontreated control.

day for 15 doses gave an increase in nonfasting glucose levels (±SE, $n = 4$) from 133.7 ± 16 mg/d in control mice to 269.4 ± 27.8 mg/d ($P < 0.05$) in the PX-866-treated mice. The treatment also gave an increase in plasma glucose $AUC_{0\text{-}180\ minutes}$ 24 hours after the last dose of PX-866, but this had recovered to control values 8 days after the last dose (Table 4). Pioglitazone significantly decreased the glucose $AUC_{0\text{-}120\ minutes}$ 24 hours after the last dose of long-term PX-866 treatment to a value not significantly different to control (Table 4).

PX-866 and Increased Neutrophils

When PX-866 was given to C57Bl/6 mice at 3 mg/kg orally every other day for 15 doses, there was a significant increase in neutrophil counts (±SE, $n = 4$) from 1.2 ± 0.3 K/μL in control mice to 3.7 ± 1.8 K/μL in PX-866-treated mice ($P < 0.05$) but with no significant change in any other blood elements. Bone marrow colony forming units showed no significant change in erythroid lineage CFU granulocyte, erythroid, macrophage, megakaryocyte; burst-forming units erythroid; or CFU erythroid and a small but significant decrease in myeloid CFU granulocyte macrophage (±SE, $n = 4$) from 388 ± 52 colonies per 60,000 bone marrow cells plated in control mice to 168 ± 59 colonies ($P < 0.05$) in the PX-866-treated mice. At the same time, there was an increase in the numbers of individual white cells in the cultures from PX-866-treated mice suggestive of altered cell adhesion.

Discussion

Sensitivity of NSCLC cell lines to growth inhibition by gefitinib is associated with inhibition of EGF-stimulated EGFR autophosphorylation, down-regulation of cell surface

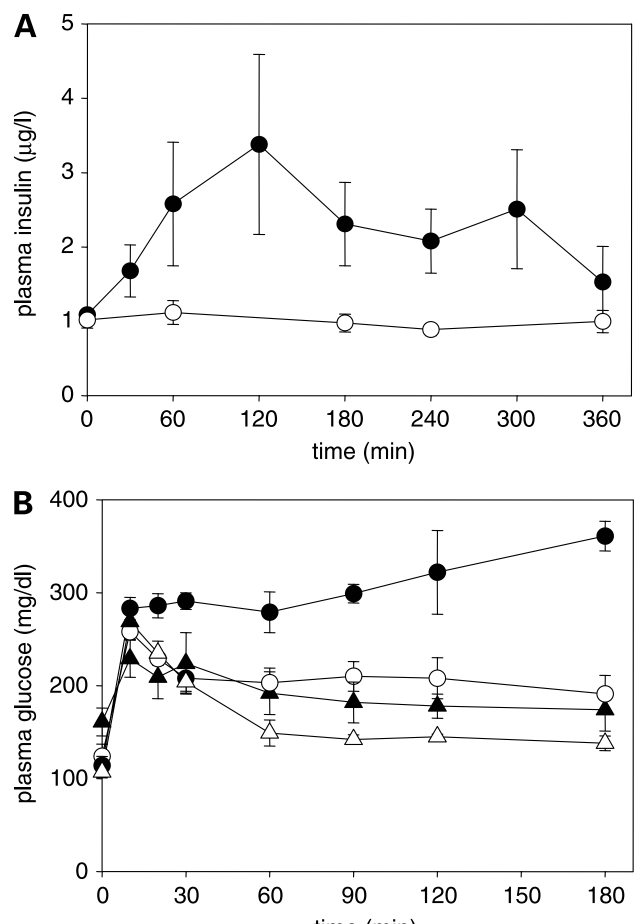

Figure 3. Effect of PX-866 on plasma insulin and glucose tolerance. **A,** plasma insulin in female C57Bl/6 mice fasted for 16 h. ○, vehicle control; ●, mice given PX-866 (10 mg/kg orally). *Points,* means of three mice; *bars,* SE. **B,** plasma glucose in female C57Bl/6 mice fasted for 16 h and given a dose of glucose of 1 g/kg orally. ○, vehicle control; ●, given PX-866 (10 mg/kg) 4 h previously; △, treated daily for 7 d with pioglitazone (10 mg/kg i.p); ▲, treated daily for 7 d with pioglitazone (10 mg/kg i.p) and given PX-866 (10 mg/kg) 4 h previously. *Points,* means of four mice; *bars,* SE.

Table 3. Toxicity of long-term PX-866 administration

Treatment group	ALT (units/L)	AST (units/L)	Glucose (mg/dL)	WBC (K/μL)	NE (K/μL)
Control	52.6 ± 13.6	142.9 ± 46.6	46.9 ± 5.1	8.9 ± 1.0	6.9 ± 0.8
PX-866 10 mg/Kg i.v.	35.5 ± 11.7	105.2 ± 19.2	76.2 ± 3.6	14.6 ± 4.2	14.0 ± 2.7
PX-866 3 mg/Kg orally	47.6 ± 16.8	152.0 ± 47.2	113.5* ± 23.4	67.8* ± 19.7	53.6† ± 10.7
PX-866 1.5 mg/Kg orally	65.6 ± 27.5	140.5 ± 35.2	100.1† ± 10.9	16.6* ± 2.4	12.5* ± 1.9

NOTE: PX-866 was administered either i.v. or orally as 14 doses every other day to male SCID mice. Twenty-four hours after the last dose, blood was collected for serum chemistry and differential blood counts. Values are the mean of four mice per group ± SE.
Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; NE, neutrophil; Hg, hemoglobin; Plt, platelet; LY, lymphocyte; MO, monocyte.
*$P < 0.5$.
†$P < 0.01$ compared with the control value.

EGFR, down-regulation of extracellular signal-regulated kinase1/2, and inhibition of PI3-K/Akt signaling (28). The PI3-K/Akt pathway is a critical pathway for cancer cell survival (29, 30). In a study by Ono et al. (28), gefitinib inhibited EGF-induced PI3-K/Akt signaling, as measured by phospho-Akt levels, in nearly all NSCLC cell lines; however, only a few lines (3 of 11) showed inhibition of phospho-Akt under serum-stimulated growth conditions. These results suggest that in many NSCLC cell lines factors other than EGF are responsible for the activation of PI3-K/Akt signaling. Tumor cells with this phenotype may show limited responsiveness to the cytostatic and/or cytotoxic activities of EGFR inhibitors. Engelman et al. (19) have recently reported that ErbB-3 couples EGFR signaling to the activation of PI3-K/Akt and that gefitinib inhibits phospho-Akt and cell growth only in NSCLC cell lines expressing EGFR, either wild type or mutant, and ErbB-3. However, forced ErbB-3 expression did not render NSCLC cells sensitive to gefitinib suggesting that pathways other than EGFR must activate the PI3-K/Akt signaling in ErbB-3-deficient cells. Other members of the ErbB receptor family may also couple with ErbB-3 to activate PI3-K and promote the cancer phenotype (19, 31). We reasoned that inhibiting PI3-K could offer a rational strategy to potentiate the antitumor activity of gefitinib in gefitinib-resistant NSCLC cell lines.

For our studies, we chose the A-549 NSCLC cell line that is among the most resistant of NSCLC lines to gefitinib (19, 28) and does not express ErbB-3 (19). PTEN deficiency can also render cells resistant to gefitinib growth inhibition presumably through constitutive activation of PI3-K/Akt signaling (32). However, genetic abnormalities of PTEN are relatively rare in lung cancer (33, 34) and A-549 cells, as do most NSCLC cell lines, has wild-type PTEN and non-constitutively activated levels of phospho-Akt (35). To inhibit PI3-K, we used PX-866 that has been shown to down-regulate tumor phospho-Akt and to exhibit antitumor activity in a number of human tumor xenograft models when given either i.v. or orally (20).

We found that PX-866 given either i.v. or orally inhibited the growth of A-549 NSCL tumor xenografts in SCID mice as effectively as gefitinib. Both agents were most active when given long-term giving tumor treated versus control values around 50%. However, when PX-866 was given together with gefitinib, A-549 tumor growth was held stationary during the first part of the treatment and increased only slightly during the later part of treatment. This was seen with both 100 mm^3 tumors and with large advanced 600 mm^3 tumors. Gefitinib failed to inhibit phospho-Akt in A-549 tumor xenografts. In the A-549 cell culture studies, gefitinib also did not inhibit phospho-Akt cells under serum-stimulated growth conditions and was only inhibitory in EGF-stimulated, serum-deprived A-549 cells. In contrast, PX-866 inhibited phospho-Akt of A-549 cells under serum-simulated growth conditions and in A-549 human tumor xenografts. Gefitinib inhibited phospho-EGFR in the A-549 human tumor xenografts and PX-866 did not. Thus, PX-866 potentiated the antitumor activity of gefitinib against even very large A-549 tumor xenografts giving complete tumor growth control in the early stages of treatment. The inhibition of tumor growth was associated with inhibition of PI3-K/Akt signaling by PX-866 and was not observed with gefitinib alone.

A previous study has reported that LY294002, a relatively toxic and nonspecific PI3-K inhibitor with limited potential for clinical development (36), given i.p. potentiates the antitumor activity of gefitinib against small (6–100 mm^3) U87:ΔEGFR human glioma cell xenografts that coexpress wild-type and mutant tumor-derived activated EGFR (37). In this study, neither gefitinib nor LY294002 showed antitumor activity alone.

The major toxicity of prolonged administration of PX-866 was hyperglycemia and decreased glucose tolerance that reversed when drug administration was stopped. Insulin signals are relayed predominantly by the PI3-K isoform p110β but also by p110α (38, 39), whereas growth signals are relayed by PI3-K p110α (40). PX-866 is a more potent PI3-K p110α inhibitor than wortmannin, but unlike wortmannin, PX-866 is a poor inhibitor of inhibitor of PI3-K p110β. Acute administration of PX-866 to mice decreased glucose tolerance at the same time that plasma insulin levels were increased suggesting a decrease in sensitivity to insulin. This is similar to the phenotype of mice deficient in the Akt2 isoform that includes marked hyperglycemia, hyperinsulinemia, and an impaired ability of insulin to lower blood glucose (41, 42). In the present study, a high dose of insulin was able to overcame the

Table 3. Toxicity of long-term PX-866 administration (Cont'd)

Treatment group	LY (K/μL)	MO (K/μL)	RBC (M/μL)	Hb (g/dL)	Plt (K/μL)	Weight change (g)
Control	1.1 ± 0.2	0.8 ± 0.1	11.0 ± 0.3	15.4 ± 0.2	1,427 ± 60	4.7 ± 0.3
PX-866 10 mg/Kg i.v.	1.8 ± 0.5	1.1 ± 0.2	10.6 ± 0.0	14.4 ± 0.1	1,390 ± 43	3.9* ± 0.2
PX-866 3 mg/Kg orally	5.2 ± 3.9	9.2 ± 3.6	10.4 ± 0.3	14.7 ± 0.4	1,665 ± 227	1.3† ± 0.5
PX-866 1.5 mg/Kg orally	3.1* ± 0.6	1.8* ± 0.2	10.3 ± 0.3	14.6 ± 0.3	1,221* ± 18	3.9 ± 0.6

increase in blood glucose caused by PX-866. Metformin, a widely used drug for the treatment of hyperglycemia of type 2 diabetes, lowers blood glucose by stimulating AMP-activated protein kinase downstream of PI3-K to increase fatty acid oxidation and to decrease triglyceride synthesis, hepatic glucose production, and glucose use (43, 44). AMP-activated protein kinase mediates the stimulation of glucose uptake through translocation of the glucose transporter-4 to the plasma membrane (45). It has been suggested that an AMP-activated protein kinase activator such as metformin might enhance tumor cell survival if used with agents such as PI3-K or Akt inhibitors that impair glucose use (46). We found that metformin had no effect on the decreased glucose tolerance caused by PX-866. It should be noted that a parallel pathway mediated by the recruitment of the Cbl proto-oncogene to the activated insulin receptor also increases glucose uptake by insulin (47).

Table 4. Effects of PX-866 on glucose tolerance in mice

Treatment	$AUC_{0\text{-}180\ min}$ (mg min mL^{-1})	$AUC_{0\text{-}180\ min}$ (mg min mL^{-1})
A. PX-866 10 mg/kg orally		
No drug	369 ± 25	533 ± 17*
Insulin 0.075 μ/kg i.p.	64 ± 25*	64 ± 5
Metformin 250 mg/kg i.p. QD × 5	367 ± 46	537 ± 4†
Pioglitazone 10 mg/kg i.p. QD × 7	274 ± 3*	340 ± 39‡
B. PX-866 3 mg/kg orally QOD × 20		
		520 ± 14*
8-d recovery		343 ± 30
Pioglitazone 10 mg/kg i.p. QD × 7		405 ± 26§

NOTE: Female C57Bl/6 mice were fasted overnight and given D(+)-glucose 1 mg/kg as a 0.1 g/mL solution orally. Blood was collected at various times up to 180 min and plasma glucose measured to obtain a plasma glucose area under the curve ($AUC_{0\text{-}180\ min}$). Mice were given PX-866 10 mg/kg orally and glucose given 4 h later, or PX-866 3 mg/kg orally every other day for 20 doses and glucose given 24 h or 8 d after the last dose. Metformin was given at 250 mg/kg daily for 5 d and pioglitazone at 10 mg/kg i.p. daily for 7 d before glucose administration. Human recombinant insulin was given at 0.075 μ/kg i.p. at the same time as glucose. Values are the mean ± SE of four mice per group.

*$P < 0.05$ compared with untreated control.

†$P < 0.05$ compared with drug-treated control without PX-866.

‡$P < 0.05$ compared with PX-866 alone.

§$P < 0.05$ compared with chronic PX-866 alone.

In contrast to metformin, the thiazolidinedione hyperglycemic drug pioglitazone reversed the inhibitory effects of both acute and chronic PX-866 administration on glucose tolerance. Thiazolidenediones sensitize the body to the metabolic effects of insulin by acting as ligands for the peroxisome proliferator-activated receptor-γ transcription factor that is present at high levels in adipose tissue (48). Peroxisome proliferator-activated receptor-γ also induces differentiation of tumor cells and its activation by pioglitazone has been reported to inhibit the growth of A-549 NSCL tumor xenograft in SCID mice (49). Whereas all the details of insulin signaling through PI3-K and the effects of glucose-lowering drugs such as metformin and pioglitazone remain to be elucidated, it seems that hypergylcemia caused by PI3-K inhibition by PX-866 is responsive to insulin and pioglitazone, which could be important for the clinical use of PX-866. The selectivity of PX-866 as an inhibitor of p110α relative to p110β, unlike wortmannin that inhibits both p110α and p110β, may also explain the more pronounced growth inhibitory effects of PX-866, and the ability of insulin and pioglitazone to reverse PX-866-induced hyperglycia.

The other pharmacologic effect of PX-866 administration was an increase in circulating neutrophils at the same time there is a decrease in bone marrow CFU granulocyte macrophage colony formation. The decrease in CFU granulocyte macrophage induced by PX-866 is consistent with the decreased sensitivity to granulocyte macrophage-colony stimulating factor observed in bone marrow–derived macrophages of $p85\alpha^{-/-}$ knockout mice (50). The increase in circulating neutrophils by PX-866 may reflect increased mobilization of progenitor cells into the peripheral circulation, perhaps associated with the decreased cell adhesion as seen in the $p85\alpha^{-/-}$ knockout mice (50).

In summary, we have shown that the PI3-K inhibitor PX-866 that shows selectivity for p110α compared with p110β potentiates the antitumor activity of the EGFR inhibitor gefitinib against even large A-549 NSCLC xenografts, with complete tumor growth control in the early stages of treatment. This therapeutic effect of PX-866 was associated with inhibition of tumor Akt phosphorylation that was not seen with gefitinib alone. The major toxicity of chronic PX-866 was a target-related hyperglycemia with a reversible decrease in glucose tolerance due to decreased sensitivity to insulin. The decreased glucose tolerance was insensitive to the AMP-activated protein kinase inhibitor metformin but was reversed by insulin and the peroxisome proliferator-activated receptor-γ activator pioglitazone. Long-term PX-866 also caused increased neutrophils counts, apparently due to vascular mobilization. Thus, PX-866 by inhibiting PI3-K/Akt signaling may have clinical use in increasing the response to EGFR inhibitors such as gefitinib in patients with NSCLC who do not respond to therapy with EGFR inhibitors.

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