

Research Article

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Bexarotene Plus Erlotinib Suppress Lung Carcinogenesis Independent of *KRAS* Mutations in Two Clinical Trials and Transgenic Models

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Abstract

The retinoid bexarotene represses cyclin D1 by causing its proteasomal degradation. The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) erlotinib represses cyclin D1 via different mechanisms. We conducted a preclinical study and 2 clinical/translational trials (a window-of-opportunity and phase II) of bexarotene plus erlotinib. The combination repressed growth and cyclin D1 expression in cyclin-E- and *KRAS/p53*-driven transgenic lung cancer cells. The window-of-opportunity trial in early-stage non-small-cell lung cancer (NSCLC) patients (10 evaluable), including cases with *KRAS* mutations, repressed cyclin D1 (in tumor biopsies and buccal swabs) and induced necrosis and inflammatory responses. The phase II trial in heavily pretreated, advanced NSCLC patients (40 evaluable; a median of two prior relapses per patient (range, 0–5); 21% with prior EGFR-inhibitor therapy) produced three major clinical responses in patients with prolonged progression-free survival (583-, 665-, and 1,460-plus days). Median overall survival was 22 weeks. Hypertriglyceridemia was associated with an increased median overall survival ($P = 0.001$). Early PET (positron emission tomographic) response did not reliably predict clinical response. The combination was generally well tolerated, with toxicities similar to those of the single agents. In conclusion, bexarotene plus erlotinib was active in *KRAS*-driven lung cancer cells, was biologically active in early-stage mutant *KRAS* NSCLC, and was clinically active in advanced, chemotherapy-refractory mutant *KRAS* tumors in this study and previous trials. Additional lung cancer therapy or prevention trials with this oral regimen are warranted. *Cancer Prev Res*; 4(6); 818–28. ©2011 AACR.

Introduction

As the leading cause of cancer-related mortality in the United States (1), lung cancer needs improved treatment and effective prevention (2). Understanding the biology of lung cancer should help in developing better clinical

strategies to control the disease (2). Uncontrolled cancer cell proliferation is associated with cell-cycle alterations (3, 4). Cyclin D1 and cyclin E regulate the G₁-S transition; prior work revealed that these cyclins are often aberrantly expressed in lung carcinogenesis (5). G₁ cyclin expression is also a negative prognostic factor in lung cancer (6–8). Given these data, the cell cycle is a promising lung cancer target (9).

The epidermal growth factor receptor (EGFR) and its ligands regulate normal and neoplastic cell growth (10). Increased EGFR expression frequently occurs in lung carcinogenesis (11). The EGFR can also regulate the cell cycle, as EGF induces cyclin D1 expression through distinct mechanisms (12–18). Treatment with the EGFR tyrosine kinase inhibitor (TKI) erlotinib causes G₁ arrest and inhibits induction of cyclin D1 in immortalized and NNK [nitrosamine-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone]-transformed human bronchial epithelial (HBE) and non-small-cell lung cancer (NSCLC) cell lines (9, 19). Erlotinib prolongs survival in some previously treated NSCLC patients (20). Lung cancers that harbor activating *EGFR* mutations are especially clinically responsive to EGFR TKIs, but EGFR wild-type tumors,

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particularly those with *KRAS* mutations, are often resistant (21–24). There is a need for improved treatments of NSCLC with or without *KRAS* or *EGFR* mutations. We previously reported results from a mechanistic window-of-opportunity trial, where we obtained pre- and post-erlotinib treatment biopsies from aerodigestive tract cancers (19). Cyclin D1 expression was a biomarker of response when therapeutic intratumoral drug levels were achieved and even in tumors with wild-type *EGFR* (19).

Retinoids (natural and synthetic derivatives of vitamin A) exert antiproliferative, differentiation-inducing, and proapoptotic effects (reviewed in ref. 25). Retinoid X receptor agonists (rexinoids) and classical retinoic acid receptor (RAR) agonists activate distinct nuclear receptors but can engage similar pathways (9, 25). Rexinoids can bypass RAR β repression, a frequent NSCLC alteration, which contributes to resistance to classical retinoids that activate RARs (2, 25). In premalignant HBE cells and in some lung cancer cell lines, the rexinoids can inhibit growth and reduce *EGFR*, phospho-*EGFR* (p-*EGFR*), and cyclin D1 expression (25–30). Bexarotene causes cyclin D1 repression via proteasomal degradation of cyclin D1 that confers G₁ arrest (26, 27). A bexarotene window-of-opportunity trial in resected NSCLC cases (30) established a nonlinear correlation between plasma and tumor concentrations of bexarotene (30). Repression of cyclin D1, *EGFR*, p-*EGFR*, and proliferation was associated with high intratumoral levels of bexarotene (30). These and other findings indicate that cyclin D1 is both a biomarker and a therapeutic target (9, 25). These data also suggest that a combination regimen that confers cyclin D1 repression would have activity against lung cancer.

Furthermore, cyclin D1 levels were much higher in NSCLC cell lines harboring *EGFR* mutations than in NSCLC cell lines expressing wild-type *EGFR* and mutant *KRAS* (31). This finding implied a link between high cyclin D1 levels and reliance on the *EGFR* pathway. Other pathways could cause high cyclin D1 expression, suggesting the promise of combining *EGFR* inhibition with a rexinoid that targets cyclin D1 for repression via a separate pathway. Cooperation between retinoid and *EGFR* pathways *in vitro* was reported (28, 32). On the basis of this cooperation, we conducted a phase I clinical trial combining erlotinib with bexarotene for advanced aerodigestive tract tumors (33). The drugs were safely combined clinically at full single-agent doses. Cyclin D1 was repressed in posttreatment versus pretreatment buccal swabs of most patients on this trial, confirming the expected biomarker response in surrogate tissues (33). Encouraging clinical results included activity in patients unlikely to benefit from erlotinib alone because their lung cancers lacked activating *EGFR* mutations (33). Trial patients who had major objective responses included non-Asian men and smokers (both of whom typically do not respond well to *EGFR* TKIs; refs. 33).

The results we report here come from new studies of bexarotene plus erlotinib in transgenic lung cancer cell lines driven by some of the same changes that are linked

to clinical lung cancer formation (e.g., *KRAS* mutations) and from 2 clinical/translational trials of bexarotene plus erlotinib, one a window-of-opportunity trial in patients with early-stage NSCLC scheduled for resection and the other a phase II trial in patients with advanced-stage, refractory NSCLC. Clinical/translational endpoints included changes in cyclin D1 expression and other biomarkers (total *EGFR*, p-*EGFR*, and Ki-67 expression) in pretreatment versus posttreatment biopsies; correlations of these changes with tumor *EGFR* or *KRAS* mutations, necrosis or inflammation in posttreatment versus pretreatment biopsies; cyclin D1 expression in posttreatment versus pretreatment buccal swabs (window of opportunity trial); and clinical tumor response rate [including data from early positron emission tomography (PET)], overall and progression-free survival (PFS), and toxicity (including hypertriglyceridemia and rash; phase II trial).

Materials and Methods

Cell culture, proliferation assays, and transgenic models

The ED1 (cyclin E^{high}) cell line was derived from a transgenic mouse that developed lung cancer after expressing wild-type cyclin E under control of the human surfactant C promoter (34, 35). The 393P lung cancer cell line was derived from *K-ras*^{LA1/+}; *p53*^{R172HΔG/+} transgenic mice (36). ED1 and 393P cells were each cultured in RPMI 1640 media supplemented with 10% FBS and 1% antibiotic and antimycotic solution at 37°C in 5% CO₂ and in a humidified incubator. For proliferation assays, ED1 (4.5 × 10³) and 393P (5 × 10³) cells were independently seeded per well of 6-well tissue culture plates in triplicates and treated 24 hours later with the indicated agents. Triplicate replicate experiments were conducted. Logarithmically growing cells were assayed 72 hours posttreatment by the CellTiter-Glo assay (Promega) and established methods (19). Erlotinib (Genentech Inc.; OSI Pharmaceuticals) and bexarotene (Ligand, Inc.; Eisai Pharmaceuticals) were each dissolved in the vehicle, dimethyl sulfoxide (DMSO). Transgenic mice were sacrificed following an Institutional Animal Care and Use Committee (IACUC)-approved protocol. Harvested lung tissues were formalin-fixed, paraffin-embedded, and sectioned for hematoxylin and eosin (H&E) and immunohistochemical staining, as before (34).

Immunoblot and immunohistochemical assays

ED1 and 393P cells were independently treated with erlotinib alone, bexarotene alone, combinations of these agents, or vehicle alone. In brief, cells were plated at the same respective densities as used in the proliferation assays. Cells were harvested 72 hours posttreatments and subjected to immunoblot analyses, as before (19). Antibodies were purchased that recognized cyclin D1 (M-20; Santa Cruz Biotechnology) or actin (C-11; Santa Cruz Biotechnology). Immunoblot assays assessed cyclin D1 and actin expression (33, 34). H&E staining was conducted as in prior work (30, 34). Immunohistochemical assays for

cyclin D1, Ki-67, total EGFR, and p-EGFR were each used (19, 30, 34) and scored (as was the histopathology) by a pathologist (V.M.), who was unaware of treatment assignments. The percent change in expression of each biomarker was scored. The percentages of tumor volumes with necrosis in posttreatment versus pretreatment biopsies were provided. The scoring system used for assessments of acute (neutrophilic infiltrate) or chronic (lymphoplasmacytic infiltrate) inflammatory responses were as follows: 0, no inflammation; 1+, 1% to 10% of tumor volume; 2+, 11% to 20% of tumor volume; and 3+, greater than 20% of tumor volume.

Patients

Eligible patients had a histologic or cytologic diagnosis of NSCLC. For the advanced stage phase II trial, eligibility included NSCLC stage IV or stage IIIB with malignant effusion, without curative treatment options, Karnofsky performance status of 60 or greater, and age older than 18 years. Prior chemotherapy or radiotherapy was allowed. Fasting triglycerides had to be below the upper limit of normal (ULN). For the window-of-opportunity trial, eligible patients had a pathologic diagnosis of NSCLC (with at least 5 unstained slides required from the pretreatment biopsy), clinical stages I, II, or IIIA, were older than 18 years, and were candidates for resection. Prior chemotherapy or radiotherapy was not allowed. Effective contraception or sexual abstinence was required for female patients of childbearing potential or male patients with female partners of childbearing potential.

Exclusion criteria were hepatic dysfunction with either bilirubin level greater than the ULN or transaminase [alanine aminotransferase (ALT) or aspartate aminotransferase (AST)] greater than 2.5 times ULN or more than 5 times ULN if there were known liver metastases; renal dysfunction (creatinine clearance <30 mL/min); and a serious uncontrolled medical disorder, or active infection, which would impair ability to receive study treatment. Patients with dementia or altered mental status prohibiting understanding or rendering of informed consent and compliance with the protocol were excluded. Prior therapeutic use of bexarotene or concurrent use of other anti-cancer approved or investigational agents were each not allowed. Previous treatment with other EGFR inhibitors was permitted.

Inclusion and exclusion criteria were assessed within 14 days before initiation of therapy, with the exception of radiographic studies, which were conducted within 28 days of screening. These clinical trials were conducted after approval by the Committee for the Protection of Human Subjects at Dartmouth College and the Institutional Review Board. Informed consent was obtained from each patient before enrollment onto these studies.

Study drugs

An Investigational New Drug (IND) application for the combination of erlotinib and bexarotene was submitted to the U.S. Food and Drug Administration (FDA) and was

granted to the principal investigator (K.H.D.). Bexarotene capsules 400 mg/m²/d orally and erlotinib 150 mg orally were taken at the same time daily. These doses were established from a phase I study (33). Treatment continued until progression of disease, unacceptable adverse effects, or withdrawal of informed consent. Atorvastatin was started for abnormal elevated fasting triglycerides or cholesterol levels. Dose modifications were allowed for toxicities from each agent. For triglyceride levels 400 to 800 mg/dL, the statin dose was increased up to the maximum as per the package insert of the agent, followed by the addition of fenofibrate. Bexarotene dose was reduced for a fasting serum triglyceride level greater than 800 mg/dL. Bexarotene dose suspensions were made for fasting serum triglyceride levels greater than 1,200 mg/dL. Once triglyceride levels decreased below 800 mg/dL, bexarotene was restarted, provided the interruption was less than 21 days.

Clinical evaluations

Patient evaluations for toxicities included physical examination and laboratory studies (complete blood cell count, comprehensive metabolic profile, and lipid profile) conducted biweekly during the first month on therapy, then monthly while on treatment, along with monthly assessment of thyroid function (TSH, T3Q, and T4). Additional tests were as clinically indicated. Radiographic evaluations by chest computed tomography (CT) were carried out every 8 weeks; responses were assessed using the RECIST (Response Evaluation Criteria in Solid Tumors) criteria. For a subset of patients, a PET-CT scan was done within 4 weeks before treatment initiation, at 10 (\pm 2) days and at 8 weeks of study therapy. PET response was based on the European Organization for Research and Treatment of Cancer criteria for PET metabolic response. An assessment was made between early response by PET and radiographic response at 2 months.

In the window-of-opportunity trial, patients received bexarotene 400 mg/m²/d orally and erlotinib 150 mg as single daily oral doses for 7 to 9 days before surgical resection and on the day of surgery. No dose modifications were permitted. Hypolipidemic therapy was not administered.

Buccal mucosal specimens

In the window-of-opportunity trial, buccal specimens were harvested on day 1 (before treatment) and on the day of surgery by swabbing the buccal mucosa, as previously described (33), except for lysis done with the Laemmli sample buffer (BioRad). Immunoblot analyses were conducted with densitometry to determine changes in cyclin D1 expression relative to actin expression as a loading control and to confirm integrity of the protein.

EGFR and KRAS mutational analyses

Genomic DNA was isolated from paraffin-embedded tissue sections of NSCLCs using the Genra PureGene Blood Kit Plus (Qiagen) and the manufacturer's recommended procedures. Samples were screened for mutations

in *EGFR* exons 19 and 21 by PCR and restriction digestion followed by capillary electrophoresis. The 7 most common *KRAS* mutations were detected using allelic discrimination probes with real-time PCR. Sanger sequencing confirmed the results, as in prior work (19, 22, 23, 33, 37, 38).

Statistical analyses

In vitro changes both in growth and in cyclin D1 expression were assessed using the 2-sample *t*-test with significance defined as a 2-sided $P < 0.05$, using Microsoft Excel software. Time to progression and overall survival were determined using the Kaplan and Meier method. The prespecified endpoints of response to treatment in the window-of-opportunity trial were defined as a 50% decrease in cyclin D1, EGFR, p-EGFR, or Ki-67 immunohistochemical expression (posttreatment versus pretreatment specimens). The prespecified number of NSCLC cases for the window-of-opportunity trial was 12. No observed responses in 12 patients would have excluded the possibility of a response rate higher than 20% with a value of $P < 0.05$ (39). The prespecified primary endpoint of the advanced stage phase II trial was radiographic response rates (including PET responses) of the combination according to the RECIST criteria. The prespecified secondary endpoints were median survival, time to progression, toxicities, and *EGFR* mutational analysis of the responding

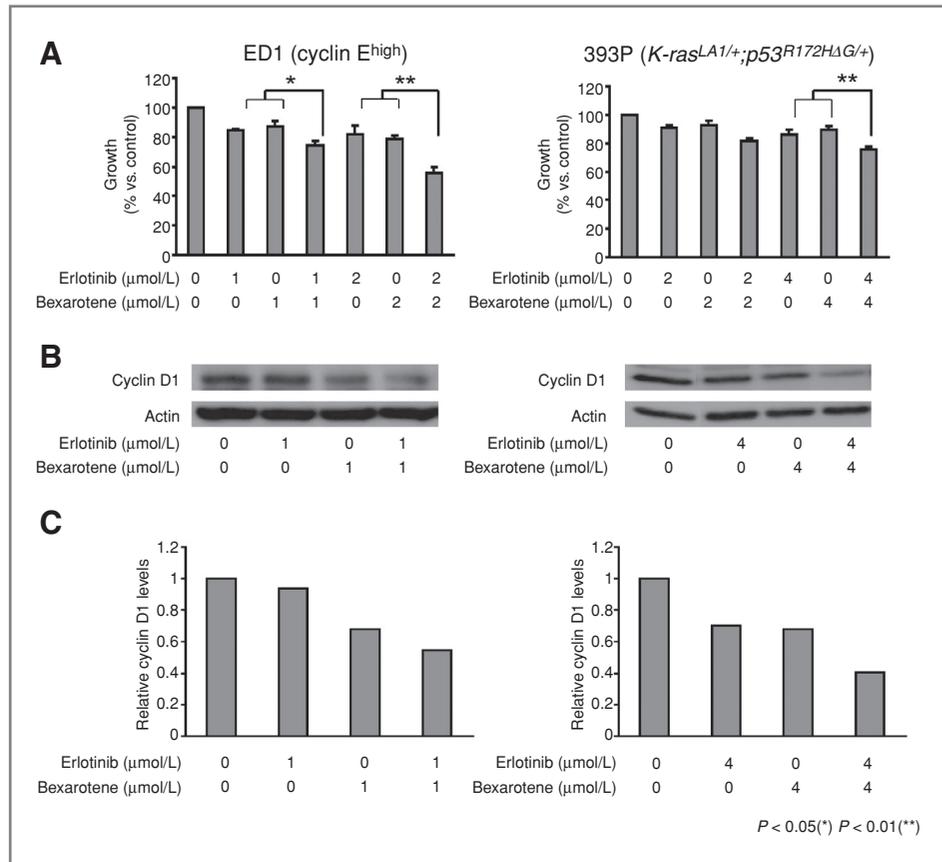
cases. The planned sample size for the advanced stage NSCLC trial was 40. No observed responses in 40 patients would have excluded the probability of a response rate of 7% or higher with a value of $P < 0.05$ (39). Associations between survival and triglyceride levels and rash were independently determined using the log-rank test done with STATA statistical suite version 10.0 (Stata Corp). Significance was defined as a $P < 0.05$.

Results

Preclinical results

KRAS mutations at codon 12, 13, or 61 were not detected in DNA sequence analysis of genomic DNA isolated from ED1 cells (data not shown), confirming that ED1 cells lacked *KRAS* activation. Drug dosages were chosen for each cell line to search for cooperative effects when bexarotene was combined with erlotinib. Significant growth repression occurred in both ED1 and 393P lung cancer cell lines after combining bexarotene with erlotinib (Fig. 1A). This repression was associated with cooperative repression of cyclin D1 protein expression in both ED1 and 393P cells (Fig. 1B; representative immunoblots). Figure 1C displays the intensities of the signals shown in Figure 1B. Results from 3 independent replicate experiments were pooled. These pooled

Figure 1. Growth response and induced changes in cyclin D1 immunoblot expression following individual or combined bexarotene and erlotinib treatments of genetically-defined murine lung cancer cells. (A) Individual and combined growth response of murine lung cancer cell lines driven by cyclin E (ED1 cells, cyclin E^{high}) or by *KRAS*/*p53* (393P cells, *K-ras*^{LA1/+}; *p53*^{R172HΔG/+}) when independently treated with the indicated dosages of bexarotene, erlotinib, or the combination. (B) Immunoblot changes in expression of cyclin D1 (relative to actin as a loading control) of a representative experiment following treatment with bexarotene, erlotinib, or the combination. (C) The intensities of the signals in panel B (relative to actin) were determined and displayed in this panel showing cooperative inhibitory effects on cyclin D1 expression. Statistically significant changes; *, $P < 0.05$; **, $P < 0.01$.



experiments confirmed the representative results shown in Figure 1B and established a statistically significant repression of cyclin D1 (data not shown).

The ED1 cell line was derived from transgenic mice expressing in the lung wild-type cyclin E; these mice develop premalignant and malignant lung lesions that recapitulate many changes found in human lung carcinogenesis (34, 35). Whether cyclin D1 was aberrantly expressed in this murine lung carcinogenesis model was examined because cyclin D1 expression is deregulated in human lung carcinogenesis (5). Cyclin D1 immunohistochemical expression increased during the progression of premalignant lung lesions to invasive or metastatic lung lesions in this murine transgenic model expressing wild-type cyclin E in the lung (Supplementary Fig. S1). This finding is consistent with a role for cyclin D1 as a biomarker and/or pharmacologic target for lung carcinogenesis. Clinical trials were conducted to explore these possibilities.

Window-of-opportunity clinical trial

Patient characteristics. We enrolled 14 early-stage lung cancer patients into this trial; 12 received study treatment, 11 underwent lung cancer resections between December 23, 2005, and June 16, 2009, and 10 were evaluable. Important features of these 10 patients included the following characteristics (Table 1).

Histopathologic, biomarker, and molecular genetic results in tumors. Ten patients had adequate pretreatment and posttreatment tissues harvested for analyses of histopathologic and biomarker responses and molecular genetic

alterations (Table 1). Of these 10 patients, 1 had an activating *EGFR* mutation at exon 21 and 5 had *KRAS* codon 12 mutations in their lung cancers; 8 had decreased cyclin D1, total EGFR, or p-EGFR immunohistochemical expression in posttreatment versus pretreatment biopsies; 6 had reduced cyclin D1 expression, 6 had a decline in p-EGFR expression, and 3 had reduced EGFR expression in immunohistochemical assays. Basal Ki-67 immunohistochemical expression was reduced in these cases, making comparisons of Ki-67 changes in pretreatment versus posttreatment biopsies inconclusive (data not shown). Of note, 8 of 10 posttreatment biopsies exhibited necrosis and evidence of chronic or acute inflammatory responses (Table 1). A single biopsy pair (patient 1) showed minimal necrosis (5%) without change in the posttreatment versus pretreatment biopsy. No pretreatment biopsies displayed acute inflammatory responses (data not shown). A single case (patient 4) had evidence of chronic inflammation in the pretreatment biopsy, but no change in this degree of inflammation was found in the posttreatment biopsy.

Biomarker responses were analyzed for associations with *EGFR* and *KRAS* mutations (Table 1). A decline in cyclin D1 and EGFR immunohistochemical expression was detected in the NSCLC case with an activating *EGFR* mutation. Of the 5 cases with *KRAS* mutations, 3 had a decrease in cyclin D1, 1 had reduced EGFR, and 2 had repressed p-EGFR immunohistochemical expression in posttreatment versus pretreatment biopsies. This high proportion of cases with biomarker and histopathologic changes (necrosis, chronic or acute inflammation) after combined bexarotene and erlotinib treatments exceeded the proportion in our prior

Table 1. Patient histopathology and biomarker characteristics in the window-of-opportunity clinical trial

Pt	Age/Gender/Smoker	Histology	EGFR	KRAS	% Reduction ^a			Necrosis %	Chronic INF ^b	Acute INF ^b
					Cyclin D1	EGFR	p-EGFR			
1	63 F (Current)	SCC	wt	wt	65	30	90	5 ^c	1+	1+
2	52 M (Never)	AD	Exon 21	wt	40	30	0	30	2+	2+
3	68 F (Former)	ADSCC	wt	mut	0	0	0	0	0	0
4	71 F (Former)	AD	wt	wt	0	0	100	10	3+ ^d	3+
5	61 F (Former)	AD	wt	mut	80	0	0	40	1+	3+
6	59 F (Current)	BAC	wt	mut	50	0	95	0	0	0
7	52 F (Current)	AD	wt	mut	0	0	0	40	3+	3+
8	63 M (Former)	BC	wt	wt	0	0	100	30	1+	3+
9	61 F (Former)	AD	wt	mut	40	65	80	20	3+	1+
10	63 M (Former)	AD	NA	NA	80	0	75	5	2+	0

Abbreviations: wt, wild-type; mut, mutant; NA, not assessed; Pt, Patients; M, Male; F, Female; SCC, squamous cell carcinoma; AD, adenocarcinoma, ADSCC, adenosquamous carcinoma; BAC, bronchioloalveolar carcinoma; BC, basaloid carcinoma; INF, inflammation.

^aThe indicated percentages reflect the proportion of biopsies with the indicated changes in the posttreatment versus pretreatment biopsies.

^bThe scoring system used to assess chronic and acute inflammatory changes in biopsy is described in Materials and Methods.

^cMinimal necrosis (5%) without change in the posttreatment versus pretreatment biopsy.

^dChronic inflammation (3+) without change in the posttreatment versus pretreatment biopsy.

window-of-opportunity trials of each agent alone (19, 30). Of note, these responses occurred whether or not *EGFR* or *KRAS* mutations were detected in the NSCLCs (Table 1). Repression of cyclin D1, total EGFR, and p-EGFR expression is shown in representative posttreatment versus pretreatment tumor biopsies (Fig. 2A). As shown in Figure 2B, no histopathologic evidence of necrosis (left panel) or evidence of necrosis (arrow, middle panel) or inflammatory responses (right panel, arrows) is apparent in representative photomicrographs of posttreatment lung cancer biopsies with these features.

Cyclin D1 expression in buccal swabs. Cyclin D1 also was assessed by immunoblot analyses of pretreatment (day 1) versus paired posttreatment (day of surgery) buccal swabs. Specimens were obtained from 6 patients, and 5 swabs were evaluable on the basis of adequate actin expression. Normalized cyclin D1 protein expression was repressed in 4 posttreatment versus pretreatment swabs (Figure 2C). The window-of-opportunity trial was well tolerated and without clinical toxicity.

Phase II clinical trial

Forty-two patients were enrolled into the advanced stage phase II trial, of which 40 received study treatment (between October 13, 2005, and February 14, 2008). Clinical characteristics of these patients (Table 2) include the following findings: 52% women; 67% with adenocarcinoma histopathology; median age of 67 years (range, 46–77); 14% current smokers and 17% never smokers; a median of 2 prior chemotherapy regimens per patient (range, 0–5); and 21% had prior EGFR inhibitor therapy.

Radiographic and metabolic responses. We evaluated radiographic responses in 19 patients, who remained on study treatment for 2 or more months. There were 2 objective responses (1 complete and 1 partial) at 2 months and a third objective response (partial) after 2 months (15.8% response rate; 95% CI: 3.4–40). Six patients had stable disease, including 1 patient with prior gefitinib therapy (35 weeks on study). Fluorodeoxyglucose (FDG)-PET-CT scans were done at baseline, at 8 to 12 days, and at 2 months of therapy in 14 patients with clinical characteristics similar to those of the entire trial cohort (data not shown). Radiographic responses at 2 months in these patients were as follows: 1 patient with complete response [early PET (at 8–12 days) showing a metabolic response], 1 patient with partial response (early PET showing metabolic progression), 2 patients with stable disease (early PET showing stable disease in 1 and progression in 1), and 10 patients with disease progressions (early PET showing stable disease in 5 and progression in 5).

Time to progression and overall survival. Patients received a median of 43 days of therapy (range, 5–1,460-plus days) and were assessed for survival in an intent-to-treat analysis. Median time to progression was 7 weeks. Median overall survival (Fig. 3A) for all analyzed patients was 22 weeks (range, 1–274-plus weeks); 22 weeks (range, 2–274-plus weeks) for the 36 patients who received more than 2 weeks of therapy; and 23 weeks

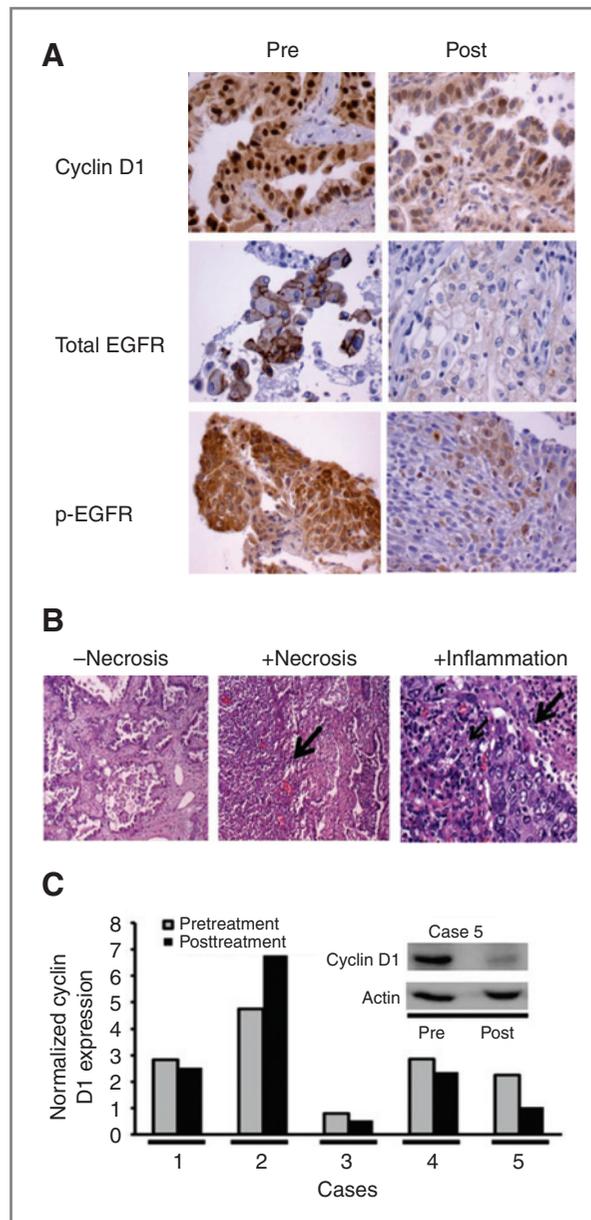


Figure 2. Biomarker, histopathologic, and buccal swab treatment effects in the window-of-opportunity trial combining bexarotene with erlotinib. A, reduced immunohistochemical expression of cyclin D1 (patient 5), total EGFR (patient 9), and p-EGFR (patient 1) in posttreatment "Post" versus pretreatment "Pre" lung cancer biopsies (200 \times). B, no evidence of necrosis ($-$ Necrosis; left), presence of necrosis ($+$ Necrosis; arrow; middle), and inflammatory response ($+$ Inflammation; small arrow shows chronic inflammatory infiltrate and large arrow shows acute inflammatory infiltrate surrounding a lung cancer; right) in representative posttreatment lung cancer biopsies (200 \times). C, cyclin D1 immunoblot expression in pretreatment versus posttreatment buccal mucosal swabs. Normalized cyclin D1 protein signals (relative to actin expression) declined in the majority of the displayed posttreatment versus pretreatment buccal mucosal swabs. The cyclin D1 immunoblot is shown for a representative case (case 5) relative to actin expression as a loading and protein integrity control.

Table 2. Clinical characteristics of patients in the phase II clinical trial ($N = 42$; $n = 40$ treated)

Median age	67 y (range, 46–77)
Gender	22 females (52%), 20 males (48%)
Race	42 white non-Hispanic (100%)
Disease stage	42 stage IV (100%) 28 adenocarcinomas (67%) 5 bronchioloalveolar type (12%) 2 squamous cell carcinomas (5%) 10 non-small cell carcinomas not otherwise specified (24%)
Tumor histology	1 mixed histology (2%) 1 non-small cell carcinoma with sarcomatoid features (2%)
Prior anti-EGFR therapy	9 (21%)
Prior chemotherapies	2 (median; range, 0–5)
Smoking status	7 never (17%), 6 current (14%), 29 former (69%)

(range, 4–274-plus weeks) for the 26 patients who received study treatment for at least 1 month.

Association of survival with triglyceridemia. Patients who developed hypertriglyceridemia in the first 4 weeks of erlotinib plus bexarotene treatment (without antilipid treatment) had an increased median overall survival (24 weeks) versus patients who did not (16 weeks). There was a statistically significant association between triglyceride levels and increased overall survival ($P = 0.001$; Fig. 3B).

Association between survival, skin rash, and clinical characteristics. Patients who developed a skin rash during therapy with erlotinib plus bexarotene had an increased median overall survival (25 weeks) versus patients who did not (14 weeks; $P = 0.03$; Fig. 3C). Combined effects of hypertriglyceridemia and rash on survival could not be assessed because of the small number of cases with normal triglycerides who did not develop rash (3 patients) versus patients with hypertriglyceridemia who developed rash (18 patients). There was no statistically significant association between triglyceride levels and the presence or absence of rash. Patients with adenocarcinoma had a longer median overall survival (22 weeks) versus patients with other histopathologic diagnoses (7 weeks; $P = 0.02$). There was no association between survival and smoking status, number of prior chemotherapies, gender, prior anti-EGFR

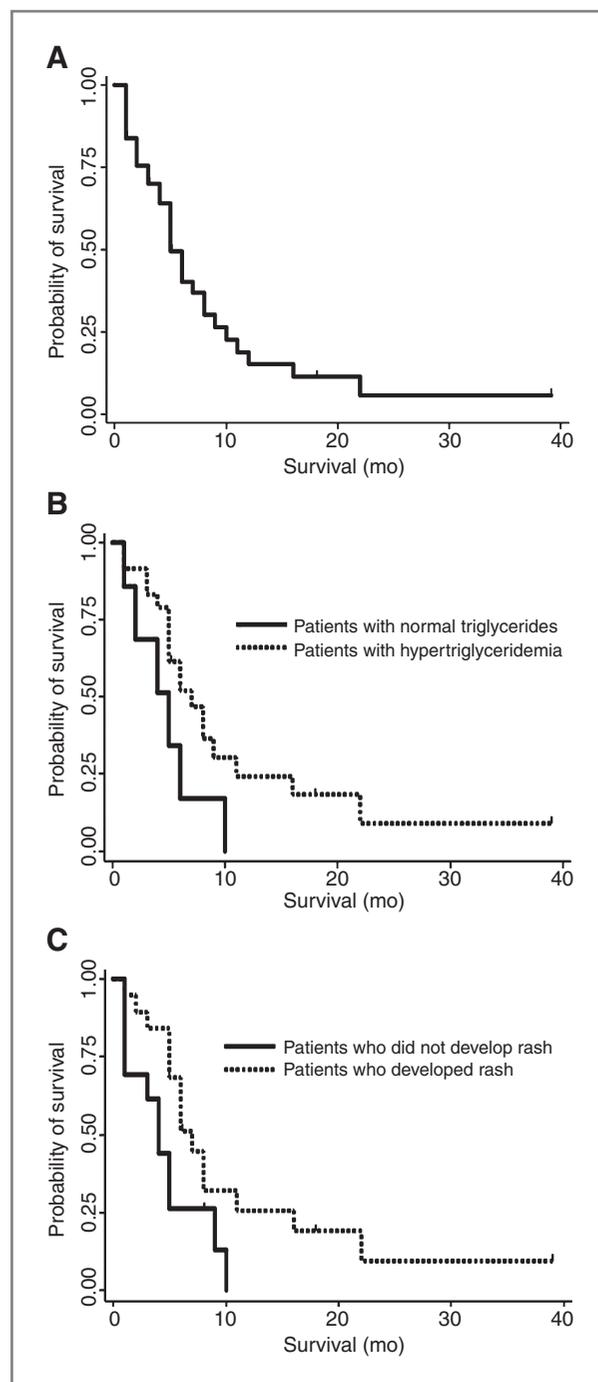


Figure 3. Kaplan-Meier survival estimates and effects of hypertriglyceridemia or rash on survival in the phase II bexarotene and erlotinib lung cancer trial. A, overall survival. Improved survival after appearance of hypertriglyceridemia (B; $P = 0.001$) or rash (C; $P = 0.03$).

therapy, cholesterol level, presence of diarrhea, or lactate dehydrogenase level. Patients with anemia or low albumin levels had decreased survival.

EGFR and KRAS mutations. We analyzed tumor samples from the 3 cases (2 females, 1 male) having the

longest PFS. The longest PFS was 1,460-plus days, which occurred for a female patient who was a never smoker, had adenocarcinoma treated with second-line therapy, had no prior EGFR inhibitor treatment, had a partial response, and is still receiving bexarotene plus erlotinib treatment of NSCLC with wild-type EGFR and wild-type KRAS. The other female patient of this subgroup had a complete response and PFS of 583 days; she was a never smoker, had first-line therapy for bronchioloalveolar carcinoma, and had an activating *EGFR* mutation at exon 21 without a *KRAS* mutation in her NSCLC. Her disease progressed after 583 days, her tumor was biopsied at this point, and she did not have the resistance mutation *EGFR T790M*. The male patient had a partial response and PFS of 665 days; he was a former smoker, had no prior EGFR inhibitor treatment, and had 2 prior therapies for adenocarcinoma with wild-type EGFR and mutant *KRAS*. This patient never progressed and died of a cause unrelated to lung cancer.

Toxicity. Clinical toxicities and laboratory abnormalities are summarized in Table 3. Most patients had hypertriglyceridemia, as expected after bexarotene treatment. There were no pancreatitis cases. Skin toxicity, manifested as dry skin, nonspecific rash, or acneiform rash, was common, generally mild, and did not require treatment interruption. There were no cases of severe diarrhea or interstitial lung disease. No cumulative toxicities were noted. Four patients died on study: none with evidence

of lung cancer progression, 2 of cardiac causes, and 2 of sepsis/multiorgan failure. Treatment was discontinued for grade 3 pulmonary hemorrhage (1 patient), mouth sores (1 patient), cough (1 patient), and abdominal pain (1 patient). One patient, who had a prior chemotherapy regimen, developed eosinophilia and fatigue at 2 weeks of treatment on bexarotene plus erlotinib. These symptoms did not improve when treatment was interrupted; the patient discontinued study participation and had a diagnosis of a hypereosinophilic syndrome within a month of stopping study treatment. This syndrome did not respond to therapy, including imatinib. This patient had received erlotinib plus bexarotene 6 months previously in the window-of-opportunity trial without adverse events and with evidence of a biomarker response (decreased cyclin D1 protein) in the resected lung cancer, which had mutant *KRAS*. Six patients had bexarotene dose reductions (4 for hypertriglyceridemia and 2 for headache). Two patients had erlotinib dose reductions for rash.

Discussion

The 3 closely interrelated studies we report here—a preclinical study comprising several analyses in transgenic models and 2 clinical/translational trials—enhanced our understanding of using bexarotene plus erlotinib for treating and, potentially, preventing lung cancer. The combination repressed growth and cyclin D1 expression in cyclin E- and *KRAS* mutation-driven transgenic NSCLC cells. The window-of-opportunity trial in early-stage NSCLC patients repressed cyclin D1 and induced necrosis and inflammatory responses including in patients with *KRAS* mutations. The phase II trial in heavily pretreated, advanced NSCLC patients produced major clinical responses (including of tumors with *KRAS* or *EGFR* mutations), with prolonged survival in 3 patients. Hypertriglyceridemia or rash significantly increased median overall survival. In summary, bexarotene plus erlotinib was active in *KRAS*-driven NSCLC cells, was biologically active in early-stage mutant *KRAS* NSCLC, and was clinically active in advanced, chemotherapy-refractory mutant *KRAS* tumors in this study and previous trials including the recently reported BATTLE trial (40), which was reported after submission of this article.

Derived from transgenic mice with lung cancers driven by high levels of cyclin E or by mutant *KRAS/p53*, the genetically defined murine lung cancer cell lines studied recapitulate common changes found in human lung cancers. Transgenic cyclin E expression also conferred aberrant expression of cyclin D1 in the lung carcinogenesis lesions that arose in these mice. This observation mimics aberrant cyclin D1 expression found in human lung carcinogenesis (5–9). We examined individual and combined treatment effects of bexarotene plus erlotinib on cyclin D1 expression and growth in these lines. Intriguingly, erlotinib plus bexarotene was significantly more active than erlotinib (or bexarotene) alone in lung cancer (19, 30, 33). When coupled with the novel findings reported here in the *KRAS*-driven transgenic lung cancer model, this is a clinically

Table 3. Adverse events

Adverse event	Grade 1/2 (%)	Grade 3/4 (%)
Rash	55	6
Triglycerides	74	13
Diarrhea	31	0
Cholesterol	32	0
Thyroxine (T4)	38	0
T3	60	0
ALT	27	3
AST	30	3
Alkaline phosphatase	24	0
Total bilirubin	8	3
Leukopenia	14	3
Hypokalemia	5	0
Hyponatremia	11	0
Eosinophilia	0	3
Hemoptysis	NA	10
Dyspnea	NA	10
Pneumonia (postobstructive)	NA	10 (8)
Pleural effusion	NA	8
Confusion	NA	10
Abdominal pain	NA	3
Acute renal failure	NA	3

Abbreviation: NA, not assessed.

relevant result. Responsive lung cancers to this regimen included those with *KRAS* mutations. Thus, this regimen seemed to broaden the clinical activity of this EGFR TKI.

Whether the clinical activity of bexarotene plus erlotinib is associated with a decline in cyclin D1 expression (or with histopathologic changes) in NSCLC with or without *EGFR* or *KRAS* mutations has not been addressed previously. The EGFR TKI erlotinib is active against advanced stage NSCLC and prolongs survival in patients, who relapse after chemotherapy, especially when rash occurs (20, 21). Bexarotene treatment also can produce clinical responses in advanced stage lung cancer (30). An early trial of bexarotene plus chemotherapy was encouraging (41). Subsequent work did not show an overall survival benefit with this regimen (42) but did show a survival advantage for patients who developed hypertriglyceridemia (42).

The studies reported here now address the link between cyclin D1 expression and effects of bexarotene plus erlotinib. The combination repressed cyclin D1 expression and induced necrotic and inflammatory changes in tumors in the window-of-opportunity trial. Cyclin D1 repression occurred in tumors with or without *EGFR* or *KRAS* mutations (Table 1). The biological activity of the combination in this trial was better than that of a previous trial of bexarotene alone in similar patients (30), in which, in addition, necrosis induction did not occur. Patients with advanced stage NSCLC had objective responses in the phase II trial. Our phase II study did not include cyclin D1 analyses, but the recently reported BATTLE trial including an arm of bexarotene plus erlotinib in similar patients did (40). The BATTLE trial found that the combination was active in mutant *KRAS* tumors and that cyclin D1 overexpression was predictive of its clinical activity (40). The clinical activity of the same regimen that was reported here was significantly associated with hypertriglyceridemia or skin rash (Fig. 3). Toxicities in the phase II trial were similar to those previously reported for single-agent bexarotene or erlotinib (19, 30). The objective responses to bexarotene plus erlotinib in heavily pretreated NSCLC patients are notable because of the rarity of clinical responses in patients with multiple prior relapses after chemotherapy (43, 44).

Prior studies showed that early FDG-PET could monitor TKI responses (45, 46). In our phase II trial, an early decline in FDG-PET activity predicted radiographic response in some cases, but we did not find an association between unchanged or increased FDG-PET activity and radiographic response at 2 months. The early increase in metabolic activity detected in a partial responder was likely a flare that was indicative of this clinical response. A larger cohort of responding cases is needed to define precisely the relationship between changes in early FDG-PET activity and response of NSCLC to bexarotene plus erlotinib.

Treatment was third line or higher for many of the NSCLC patients entered onto the phase II trial. On the basis of historical controls, the expected overall survival in similar patients treated with chemotherapy is 4 or fewer months (43, 44). With a median overall survival of 5.5

months for all treated patients and of 6 or more months for patients who developed hypertriglyceridemia or rash (Fig. 3), our present phase II trial provides evidence for the clinical activity of bexarotene plus erlotinib. The combined results from this and our prior phase I trial of the same regimen (33) show that patients with elevated triglycerides had a median survival of 24 weeks (range, 1–274-plus) versus 21 weeks (range, 4–72) for patients with normal triglycerides ($P = 0.008$). Future work should explore the mechanistic basis for this beneficial association.

The lung cancer patients in the phase II trial with the longest PFS and overall survival had features that differed intriguingly from patients who responded in prior NSCLC EGFR TKI trials, where EGFR TKI treatment increased survival of patients with activating *EGFR* mutations in their NSCLC (22, 23, 47). Of the 3 longest survivors in our present trial, only 1 had an activating *EGFR* mutation in the tumor. Another of the longest survivors had NSCLC with a *KRAS* mutation, which is linked to a poor response to EGFR inhibition in lung and many other cancers (24, 47, 48). The third longest-term survivor had neither an *EGFR* mutation nor a *KRAS* mutation in the lung tumor. With these collective findings of activity in NSCLC with or without *EGFR* or *KRAS* mutations, bexarotene plus erlotinib potentially can address the medical need of NSCLC patients with *KRAS* mutations and wild-type EGFR, whose need is not met by EGFR inhibitors or other chemotherapies.

Our recent work revealed that cyclin-dependent kinase-2 (CDK-2) inhibition is active against NSCLC cells even with *RAS* mutations (49). Perhaps incorporating a CDK-2 inhibitor into a cyclin D1-targeting regimen would enhance clinical activity against lung cancer including those with *KRAS* mutations. Future clinical work should definitively examine whether combining bexarotene with erlotinib has activity against mutant *KRAS* lung cancer.

Often deregulated in lung carcinogenesis, cyclin D1 and EGFR are targets for lung cancer therapy and chemoprevention (9, 25). Erlotinib and bexarotene are taken orally, tolerable in combination, and thus attractive candidates for clinical lung cancer chemoprevention within optimized populations. Before launching large, costly clinical cancer chemoprevention trials of the combination; however, investigators need to confirm that pathways engaged *in vitro* are also activated in relevant *in vivo* models. Relevant pathway activation has been seen in mechanistic studies of the effects of EGFR TKI or rexinoid treatments in preventing carcinogen-induced lung cancer in mice (50). These findings should be extended to the use of this combination regimen in clinically predictive mouse lung cancers models. Activity in these models would provide a further rationale to use the same regimen for clinical lung cancer chemoprevention in the optimal patient population.

The encouraging activity of this regimen includes (a) suppression of cyclin D1 and cell growth in *KRAS*-driven lung cancer cells, (b) suppressed cyclin D1 expression and

induced necrosis in short-term therapy of early-stage NSCLC (including patients with *KRAS* mutations), and (c) activity in advanced chemotherapy-refractory NSCLC in our prior phase I trial, current phase II trial, and the recently reported BATTLE trial. These translational research results warrant further study of bexarotene plus erlotinib for lung cancer therapy or chemoprevention.

Disclosure of Potential Conflicts of Interest

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Cancer Prevention Research

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