# Phosphorylation of extracellular signal-regulated kinase 1 and 2, protein kinase B, and signal transducer and activator of transcription 3 are differently inhibited by an epidermal growth factor receptor inhibitor, EKB-569, in tumor cells and normal human keratinocytes

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## Abstract

**EKB-569 is an irreversible inhibitor of epidermal growth factor receptor (EGF-R) tyrosine kinase. It inhibits EGF-induced phosphorylation of EGF-R and the growth of tumors that overexpress EGF-R in animal models. In clinical trials, EKB-569 and all other EGF-R inhibitors cause skin rashes. To understand the latter phenomenon, the effect of EKB-569 on EGF-R as well as downstream signaling to phosphoinositide 3-kinase-protein kinase B (AKT), extracellular signal-regulated kinase 1 and 2 (ERK1/2), or signal transducer and activator of transcription 3 (STAT3) pathways were compared in tumor cell lines and normal human keratinocytes (NHEK) grown in tissue culture. Tumor cell lines that have high (A431 epidermoid and MDA-468 breast carcinomas) and low (MCF-7 breast carcinoma) expression of EGF-R were used. NHEK cells express at least 15-fold less EGF-R than A431 cells. EKB-569 was a potent inhibitor of proliferation in NHEK, A431, and MDA-468 cells ($IC_{50}$ = 61, 125, and 260 nM, respectively) but not MCF-7 cells ($IC_{50}$ = 3600 nM). EKB-569 was also a potent inhibitor of EGF-induced phosphorylated EGF-R (pEGF-R) in A431 and NHEK cells ($IC_{50}$ = 20–80 nM). The reduction in pEGF-R paralleled inhibition of phosphotyrosine-705 STAT3, while the inhibition of phosphorylated AKT and phosphorylated ERK1/2 occurred at higher concentrations of EKB-569 (75–500 nM) in both A431 and NHEK cells. The effects were specific because EKB-569 did not inhibit the nuclear factor-κB pathway. It is proposed that skin toxicity associated with EKB-569 is due to inhibition of EGF-R signaling. Downstream signal transduction markers, particularly the activation status of STAT3, may be useful surrogate markers to guide clinical development of EGF-R inhibitors. [Mol Cancer Ther. 2004;3(1):21–27]**

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## Introduction

The epidermal growth factor receptor (EGF-R) is a 170-kDa glycoprotein containing an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain (1). On binding ligands, such as EGF or transforming growth factor-α (TGF-α), EGF-R dimerizes with itself (homodimerization) or other members of the family such as c-*erb*B-2 (heterodimerization). Tyrosine kinase activity increases and the receptor phosphorylates tyrosine residues on itself (autophosphorylation). Phosphorylated EGF-R (pEGF-R), like other activated receptor tyrosine kinases, phosphorylates and activates several signal transduction pathways downstream of EGF-R, including phosphoinositide 3-kinase-AKT, extracellular signal-regulated kinase 1 and 2 (ERK1/2), and signal transducer and activator of transcription 3 (STAT3) pathways that ultimately control cell proliferation (1, 2).

Because many solid tumors, including those derived from the head, neck, lung, bladder, breast, and prostate, have hyperactivated EGF-R (3, 4), there has been great interest in the use of EGF-R inhibitors to control cancer. Two neutralizing antibodies directed at the extracellular region of the EGF-R are in phase I–III trials (1, 5). Numerous small molecule inhibitors of EGF-R kinase are in phase I–III trials (1). The EGF-R inhibitor studied here is a 3-cyanoquinoline, designated as EKB-569 (6). It is a potent, irreversible inhibitor of EGF-R kinase that inhibits the growth of tumors that overexpress EGF-R in animal models and is in phase I trials (7).

EGF-R also plays an important role in the regulation of epidermal maintenance and development. This claim is supported by numerous findings. First, EGF-R is expressed in the basal layer of the epidermis and outer root sheath of hair follicle, the same region that contains proliferating keratinocytes (8). Second, genetic manipulation of the EGF-R signal transduction system can produce alteration in the skin. For example, wavy coat and curly whiskers are found in mice deficient for TGF-α (9, 10). The same phenotype is found in a naturally occurring mouse mutant strain called waved-2, which contains a point mutation in the kinase domain of EGF-R resulting in reduced kinase activity (11, 12). In addition, deletion of EGF-R blocks skin papilloma development in transgenic mice expressing a dominant-negative form of Son of Sevenless in keratinocytes (13), while transgenic mice overexpressing TGF-α display thickening of the skin and may develop papillomas (14, 15). Third, skin acneiform rashes occur in patients treated with small molecule inhibitors of EGF-R (1) including EKB-569 (7) or the c225 neutralizing

antibody to the receptor (16, 17). Fourth, several EGF-R ligands are secreted by normal keratinocytes (18). Finally, EGF-R is one of the major regulators of keratinocyte motility (19).

Based on these observations, we hypothesized that EKB-569 blocks EGF-R activation in keratinocytes as well as signal transduction pathways downstream of EGF-R. The following data demonstrate that specific signal transduction pathways are coordinately inhibited by EKB-569 in normal human keratinocytes and human tumor cell lines. These transduction signaling pathways, also inhibited in parallel in a human tumor overexpressing EGF-R that are grown in mice, may be useful as surrogate markers for the action of EKB-569 in patients.

## Materials and Methods

### Materials

EKB-569 was synthesized by Wyeth Research Chemical Sciences, Pearl River, NY (20). Human recombinant EGF and human recombinant TGF-α were obtained from Biosource (Camarillo, CA). Human recombinant tumor necrosis factor-α (TNF-α) was obtained from Sigma Chemical Co. (St. Louis, MO). A431, MCF-7, and MDA-468 cells were obtained from the American Type Culture Collection (Rockville, MD). Proliferating NHEK cells from adult keratinocytes were obtained from Clonetics (Walkersville, MD). Tumor cells were maintained as previously specified (6). NHEK cells were maintained in complete keratinocyte growth medium (basal medium) containing 0.15 mM calcium, 0.1 ng/ml human recombinant EGF, 5.0 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, and 7.5 mg/ml bovine pituitary extract (supplied by Clonetics).

### Cell Proliferation Assay

To determine the effect of EKB-569 on the growth of human tumor cell lines and NHEK cells, proliferation in 96-well dishes was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (21). For experiments with A431, MCF-7, and MDA-468, $3 \times 10^3$ cells/well were seeded in 100 μl complete DMEM/10% fetal bovine serum for A431 and MDA-468 cells or complete improved MEM/20% fetal bovine serum for MCF-7 cells. For experiments with keratinocytes, $3 \times 10^3$ cells/well were seeded in 100 μl complete keratinocyte growth medium. After 2 h, EKB-569 was added in 100 μl (0.001–10 μM) in triplicate and incubated at 37°C. After incubation for 5 days, the medium was removed from each well and fresh medium (150 μl) + 1 mg/ml MTT solution (50 μl) was added. After incubation for 2 h at 37°C, the medium was replaced with 150 μl DMSO, and absorbance at 540 nm in each well was determined. The $IC_{50}$ was calculated by linear regression of the data.

### Inhibition of Phosphorylation and Immunoblot Analysis

A431 and MDA-468 cells were seeded ($1 \times 10^6$) into six-well dishes 24 h in complete medium and then preincubated in serum-free medium for 1 day prior to use. NHEK cells were seeded at $9 \times 10^5$ cells/well 5 days prior to 24 h preincubation in serum-free medium. Cells were then treated with no drug or varying concentrations of EKB-569 for 2 h prior to coincubation with 10 or 100 ng/ml EGF or TGF-α for 15 min. In some experiments, cells were also treated for 8 h with varying concentrations of EKB-569 in serum-containing media. After this period, cell lysates were prepared as described previously (6). Briefly, cells were washed twice with cold PBS before adding lysis buffer [10 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml pepstatin A, 10 mg/ml leupeptin, 20 kIU/ml aprotinin, 2 mM sodium orthovanadate, and 100 mM NaF] for 20 min on ice. Cell lysates were then centrifuged at 14,000 rpm in a microcentrifuge (10 min, 4°C) before resolving the protein by SDS-PAGE. Proteins within the gels were transferred to polyvinylidene difluoride (PVDF) membrane and blots were probed with specific antibodies. The following dilution of each antibody was used to detect the protein of interest: EGF-R and IκBα (polyclonal antibodies; Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:500 and 1:1000 dilutions, respectively); AKT, ERK1/2, and STAT3 (polyclonal antibodies; Cell Signaling, Beverly, MA; 1:1000 dilution); pEGF-R (anti-phosphotyrosine antibody conjugated with horseradish peroxidase; BD Transduction Laboratories, San Diego, CA; 1:1000 dilution); phosphorylated AKT (pAKT; anti-pAKT polyclonal antibody; Cell Signaling; 1:1000 dilution); phosphorylated ERK1/2 (pERK1/2; anti-pERK1/2 polyclonal antibody; Promega, Madison, WI; 1:2500 dilution); phosphotyrosine-705 STAT3 (pSTAT3-$Y^{705}$; anti-pSTAT3-$Y^{705}$ polyclonal antibody; New England Biolabs, Beverly, MA; 1:1000 dilution); and phosphoserine-727 STAT3 (pSTAT3-$S^{727}$; anti-pSTAT3-$S^{727}$ polyclonal antibody; Cell Signaling; 1:1000 dilution). The secondary antibody, when needed, was a goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA) used at 1:3000 dilution. The signal was developed using the enhanced chemiluminescence method (Amersham, Piscataway, NJ). The resultant film was subjected to quantitative analysis using a densitometer (Fluor-S MultiImager, Bio-Rad). The percent inhibition of EGF-R tyrosine phosphorylation was measured by calculating the signal intensity of pEGF-R in cells treated with EGF alone *versus* those treated with EGF + EKB-569. Similar measurements were made for other proteins. Note that the level of pEGF-R can be easily monitored with the nonspecific antiphosphotyrosine antibody because it is a prominent band at 170 kDa that is modulated by EGF.

## Results

### EKB-569 Inhibits Cell Proliferation of Tumor Cells That Overexpress EGF-R

The effects of EKB-569 on cells that highly overexpress EGF-R were compared with cells that express lower levels of EGF-R (Fig 1). A431, MDA-468, and MCF-7 cells, which have $2.0–2.5 \times 10^6$ (22), $1.9 \times 10^6$ (23), and $1 \times 10^4$

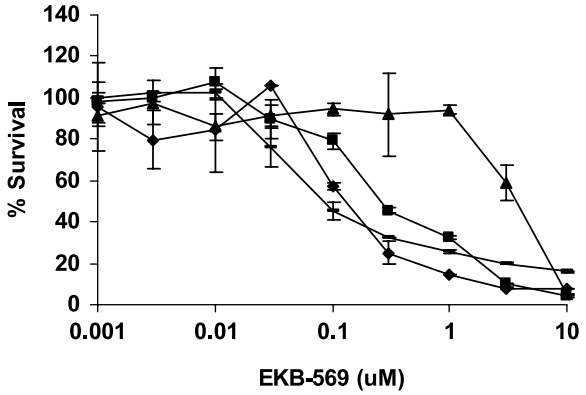


**Figure 1.** EKB-569 inhibits cell proliferation of cells that overexpress EGF-R and NHEK, cells with a low content of EGF-R. A431 (♦), MDA-468 (■), and MCF-7 (▲) cells and keratinocytes (–) were seeded at 3000 cells/well 2 h before adding EKB-569. Incubation with EKB-569 was for 5 days. Cell survival was determined by the MTT assay and expressed as percent survival compared with untreated cells.

(23) EGF-R molecules/cell, respectively, were used. Cells were grown in 96-well dishes in the presence of increasing concentrations of EKB-569 for 5 days. EKB-569 inhibited the proliferation of A431 and MDA-468 cells in a dose-dependent manner, with $IC_{50}$ = 125 and 260 nM, respectively. The MCF-7 cells were considerably less responsive ($IC_{50}$ = 3600 nM). The $IC_{50}$ for EKB-569 in A431 cells is in close agreement with a previously published value (80 nM; 20). It has previously been reported that a cell line with low expression of EGF-R or HER2 (SW-620 colon carcinoma) requires ~10-fold more EKB-569 to inhibit cell growth (20) and is consistent with the low potency of EKB-569 observed in MCF-7 cells.

### EKB-569 Is a Potent Inhibitor of NHEK Cell Growth

The effects of EKB-569 on tumor cells were compared with the effects observed on NHEK in tissue culture. Using immunoblot assay, the level of EGF-R in keratinocytes was estimated to be ~15-fold lower than that found in A431 cells or $1.3 \times 10^5$ receptors/cell; this is in agreement with previous studies (24). Surprisingly, EKB-569 was a potent inhibitor of the growth of NHEK cells; the $IC_{50}$ was estimated to be 61 ± 18 nM (mean ± SE; $n$ = 3; Fig. 1). The sensitivity to EKB-569 was unexpected because tumor cells that have low EGF-R levels (*i.e.*, MCF-7 cells) require 10-fold more EKB-569 to inhibit cell growth.

### Effect of EKB-569 on the Activation of EGF-R, AKT, ERK1/2, and STAT3 in A431 Cells after EGF Induction

Because receptor autophosphorylation is the earliest step in the signal transduction pathway by which EGF activates cells, we examined the effect of EKB-569 on EGF-induced EGF-R phosphorylation (in serum-free medium) as well inhibition of pEGF-R in serum-containing medium. The effect of EKB-569 on EGF-induced pEGF-R was studied with 10 or 100 ng/ml EGF. EKB-569 inhibited EGF-induced pEGF-R (Fig. 2; Table 1). If 10 or 100 ng/ml EGF were used, the $IC_{50}$ for EKB-569 were 20 and 40 nM, respectively. When induction of phosphorylation was done with 10 ng/ml EGF, EKB-569 inhibition of pAKT and pERK1/2 closely paralleled the inhibition of pEGF-R (Fig. 2). However, if 100 ng/ml EGF induction was used, the $IC_{50}$ for inhibition of pAKT and pERK1/2 occurred at concentrations of EKB-569 that were 2–8-fold higher than those needed to inhibit pEGF-R. In contrast, pSTAT3-$Y^{705}$ inhibition closely paralleled the effects on pEGF-R regardless of the amount of EGF used (Fig. 2, B and C). Because maximal transcription activation by STAT3 also requires serine phosphorylation (25), we also measured inhibition of pSTAT3-$S^{727}$ after inducing A431 cells with 10 ng/ml EGF. Under these conditions, the inhibition of pSTAT3-$S^{727}$ was also associated

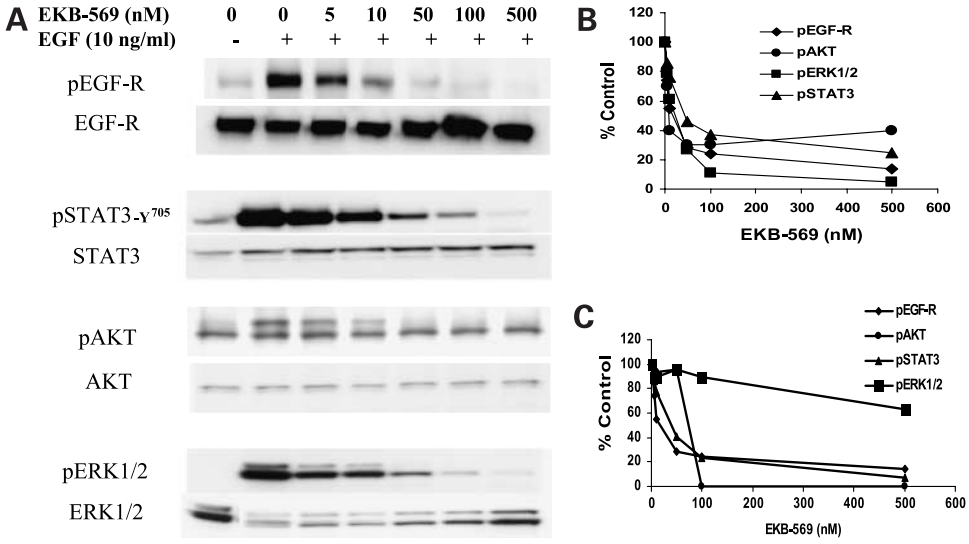


**Figure 2.** EKB-569 inhibits EGF-induced pEGF-R, pAKT, pERK1/2, and pSTAT3 in A431 cells. A431 cells were seeded into plates and incubated with no drug or varying concentrations of EKB-569 for 2 h before coincubation with 10 ng/ml (**A** and **B**) or 100 ng/ml (**C**) EGF for 15 min. After this period, cell lysates were prepared and proteins were resolved by SDS-PAGE. Gels were transferred to PVDF membrane and blots were probed with specific antibodies.

**Table 1. Effect of EKB-569 on the inhibition of activation of EGF-R and its downstream signal transduction pathways**

| Cell Line | EGF (ng/ml) | TGF-α (ng/ml) | Serum | $IC_{50}$ (nM) | | | |
|---|---|---|---|---|---|---|---|
| | | | | pEGF-R | pSTAT3-$Y^{705}$ | pERK1/2 | pAKT |
| A431 | 10 | | | 20 | 45 | 25 | 10 |
| | 100 | | | 40 | 45 | 300 | 75 |
| | | | + | 200 | 200 | 80 | >500 |
| MDA-468 | 10 | | | 210 | 190 | 270 | >500 |
| | | | + | 160 | 125 | >500 | >500 |
| | | 10 | | 360 | 400 | 250 | >500 |
| Keratinocytes | 10 | | | 80 | 70 | >500 | 440 |
| | 100 | | | 25 | 30 | 300 | 290 |
| | | | + | 80 | >1000 | 80 | NA[a] |
| | | 10 | | 56 | 60 | 62 | >100 |

[a]Not applicable or no induction of pAKT observed.

with the inhibition of pEGF-R (data not shown). No significant change in the amount of protein expression was observed for EGF-R, AKT, and STAT3 in these experiments, although inconsistent changes were observed in some experiments for ERK1/2.

### Effect of EKB-569 on the Activation of EGF-R, AKT, ERK1/2, and STAT3 in MDA-468 Cells after EGF Induction

In MDA-468 cells, the effect of EKB-569 on EGF-induced EGF-R tyrosine phosphorylation (pEGF-R) was studied with 10 ng/ml EGF.

Under this condition, the inhibition of EGF-induced pEGF-R and pSTAT3-$Y^{705}$ occurred at similar concentrations of EKB-569 ($IC_{50}$ = 210 and 190 nM, respectively). The inhibition of activated AKT and ERK1/2 occurred at high concentrations of EKB-569 (270 ≥ 500 nM; Table 1). No change in protein expression for any marker was observed (data not shown).

### Inhibition of Activated EGF-R and STAT3 in NHEK Parallels Inhibition Seen in A431 Cells after EGF Induction

The effects of EKB-569 on pEGF-R and pathways downstream of EGF-R were examined in keratinocytes. Similar to A431 and MDA-468 cells, EKB-569 inhibited pEGF-R induced by 10 or 100 ng/ml EGF in keratinocytes ($IC_{50}$ = 80 and 25 nM, respectively; Fig. 3; Table 1). These results suggest that NHEK cells are highly sensitive to EKB-569 and could be a good surrogate cell type to study the effects of EGF-R inhibitors on activated EGF-R. Furthermore, the results may help to explain the basis of skin toxicity observed after patients are treated with EGF-R inhibitors such as EKB-569.

As with A431 cells, signal transduction pathways that are downstream of EGF-R were also affected in the same manner by EKB-569 in NHEK cells. In particular, EKB-569 inhibited EGF (10–100 ng/ml)-induced pSTAT3-$Y^{705}$

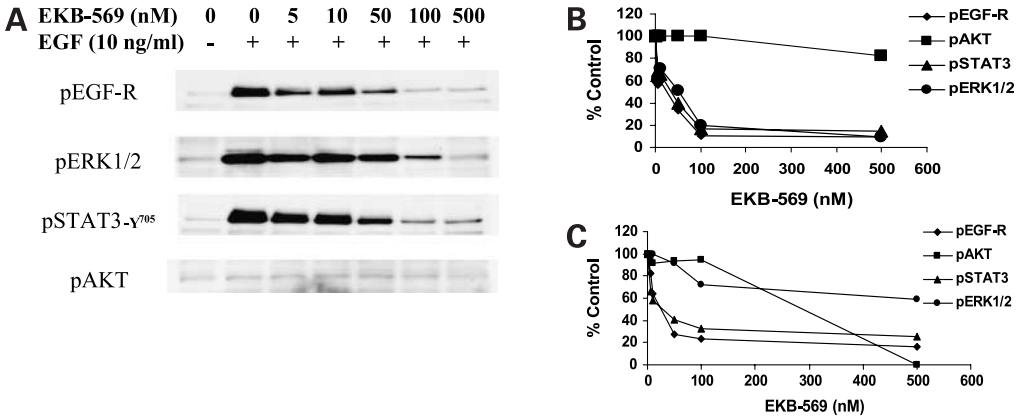


**Figure 3.** EKB-569 is a potent inhibitor of EGF-induced pEGF-R and pSTAT3 in NHEK cells. Keratinocytes (NHEK cells) were seeded and incubated with no drug or different concentrations of EKB-569 for 2 h after overnight incubation with EGF-free medium. Cells were then induced with 10 ng/ml (**A** and **B**) and 100 ng/ml (**C**) EGF. After 15 min, cell lysates were prepared and proteins were resolved by SDS-PAGE. Gels were transferred to PVDF membrane and blots were probed with specific antibodies (shown in *left panel* for 10 ng/ml EGF induction). **B** and **C**, quantitative analysis of results. The total level of EGF-R, AKT, ERK1/2, and STAT3 was not changed.

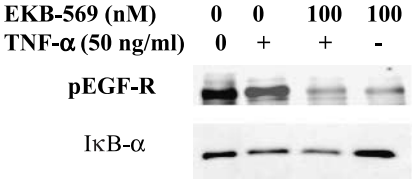


**Figure 4.** EKB-569 does not inhibit IκBα. A431 cells were seeded in serum-containing medium and incubated without or with 100 nM EKB-569 for 2 h. Cells were then treated for 15 min with or without 50 ng/ml TNF-α. Cell lysates were prepared and blots were probed for pEGF-R or IκBα expression.

($IC_{50}$ = 70–30 nM) similar to the concentrations of the agent needed to inhibit pEGF-R ($IC_{50}$ = 80–25 nM), while the inhibition of pAKT and pERK1/2 occurred at higher concentrations of EKB-569 ($IC_{50}$ = 290 ≥ 500 nM; Fig. 3; Table 1).

### Effect of EKB-569 on NHEK and MDA-468 Cells after TGF-α Induction

EGF-R has numerous ligands including EGF and TGF-α (1). Because TGF-α is thought to be one of the principle ligands for EGF-R in the skin (18, 22), the effects of EKB-569 on pEGF-R and pathways downstream of EGF-R were also examined after TGF-α stimulation. Initially, the amount of TGF-α was optimized by examining the stimulation of EGF-R after cells were given 5–250 ng/ml TGF-α. Although 5 ng/ml TGF-α was sufficient to induce strong stimulation in NHEK cells, in MDA-468 cells, induction was stronger with high concentrations (10–250 ng/ml; data not shown). Therefore, for these experiments, we used 10 ng/ml TGF-α for 15 min to stimulate the EGF-R signal transduction pathway.

In NHEK cells, EKB-569 was a potent inhibitor of TGF-α-mediated EGF-R activation ($IC_{50}$ = 56 nM) and was associated with inhibition of pSTAT3-$Y^{705}$ and pERK1/2 ($IC_{50}$ = 60 and 62 nM, respectively). MDA-468 cells were less responsive to EKB-569-mediated inhibition of TGF-α-induced signaling ($IC_{50}$ = 360, 400, and 250 nM for pEGF-R, pSTAT3-$Y^{705}$, and pERK1/2, respectively).

### Effect of EKB-569 on EGF-R and Downstream Markers in A431 and MDA-468 Cells and Keratinocytes in Serum-Containing Medium

The experiments described above were done in serum-free medium so that the effect of growth factors could be assessed. However, in the physiological setting, cells grow in the presence of serum. To more closely approximate the clinical setting, the effect of EKB-569 on cells grown in complete (serum-containing) medium was also examined. It was found that in A431 cells 200 nM EKB-569 treatment for 8 h was needed to inhibit pEGF-R and pSTAT3-$Y^{705}$ by 50%. In MDA-468 cells grown under the same conditions, 160 and 125 nM EKB-569 caused 50% inhibition of activated EGF-R and STAT3, respectively (Table 1). In keratinocytes, the $IC_{50}$ for pEGF-R inhibition was 80 nM, which is lower than the one observed for A431 and MDA-468 and not associated with pSTAT3-$Y^{705}$ inhibition ($IC_{50}$ > 1000 nM). The inhibition of pEGF-R was instead associated with pERK1/2 inhibition ($IC_{50}$ = 80 nM). The latter result agrees with a study reporting dramatically reduced levels of pERK1/2 and cell proliferation in basal keratinocytes after treatment with the EGF-R neutralizing antibody, IMC-c225 (24).

### Effect of EKB-569 on EGF-R and IκBα Kinase Signaling Pathways in A431 Cells

To determine if the inhibitory effect of EKB-569 was specific to the EGF-R signal transduction pathway, we studied the effect of EKB-569 on IκBα, a molecular marker for the IκBα kinase (IKK)-mediated signal transduction to nuclear factor-κB (NF-κB; 26, 27). Therefore, we incubated A431 cells (grown in serum-containing media) in the presence of 100 nM EKB-569 for 2 h followed by exposure to TNF-α, a ligand known to stimulate the NF-κB pathway. The concentration of EKB-569 was chosen because it was close to that needed to cause 50% inhibition of growth in this cell line. After 15 min incubation with TNF-α, we measured EGF-R phosphorylation and IκBα expression. We observed that the IKK pathway was active in A431 cells because, as expected (26), TNF-α induced a decrease in IκBα expression. However, 100 nM EKB-569 did not alter IκBα expression in the presence or absence of TNF-α, while the compound caused inhibition of EGF-R phosphorylation (Fig. 4). These results indicate that, under these conditions, EKB-569 specifically inhibits the EGF-R signaling pathway.

## Discussion

The present study demonstrates that the EGF-R-mediated signal transduction pathway in keratinocytes is similar to A431 tumor cells (Table 1). In addition, the EGF-R inhibitor, EKB-569, specifically inhibits EGF- or TGF-α-mediated signal transduction in a similar manner in these cell lines. Therefore, NHEK cells could be a good cell type to investigate the mechanism of action of EGF-R inhibitors. This would be particularly relevant as skin rashes have been reported when patients are treated with either neutralizing antibodies to EGF-R or small molecule (kinase) inhibitors of the protein (1).

It has previously been shown in skin biopsies from patients treated with an EGF-R neutralizing antibody (IMC-c225) that the levels of pERK1/2 levels and cell proliferation (as assessed by Ki67 immunoreactivity) were dramatically reduced in basal keratinocytes (24). The authors conclude that pERK1/2 status may be useful in determining the activity of EGF-R inhibitors. Consistent with this, it has recently been shown that a small molecule inhibitor of EGF-R kinase, ZD1839, inhibited ERK1/2 phosphorylation in immortalized keratinocytes and cutaneous squamous cancer cells (28). We suggest here that pSTAT3-$Y^{705}$ should also be considered a useful surrogate marker for EKB-569 activity because the inhibition of pEGF-R by EKB-569 closely parallels the inhibition of pSTAT3-$Y^{705}$ in A431 cells and normal keratinocytes (Table 1) grown under most experimental conditions used here. It is known that STAT3 is hyperactivated in many types of cancers and aberrant activation of the protein leads to malignancies (29). Beyond this, STAT3 but not STAT1

activation is required for EGF-R-mediated cell growth in squamous epithelial cells (30). In addition, tyrosine phosphorylation of STAT constitutes an early event in the activation of these transcription factors required for their dimerization and DNA binding activity. It is also important to note that phosphorylation of a serine residue in the transactivation domain of STAT1 and STAT3 enhances the transcriptional activity of these STATs (30). The implication is that both tyrosine phosphorylation and serine phosphorylation are essential for full activation STAT signaling and that STATs are points of convergence for tyrosine and serine kinases (25).

In these studies, and consistent with a previous report (31), we also found that EKB-569 was a potent inhibitor of pSTAT3-$S^{727}$ and that this inhibitory effect paralleled the inhibition of EGF-R phosphorylation. Clinical procedures that estimate the activity of pSTAT3 in tumor samples have been developed (32) and may be useful for further studies with EKB-569. These data further support the possibility that interference with STAT signaling may have therapeutic value (29).

In contrast to pSTAT3-$Y^{705}$, high concentrations of EKB-569 (100–500 nM) are needed to inhibit EGF-induced activation of AKT and ERK1/2 in both A431 and NHEK cells (Table 1). This suggests that pAKT and pERK1/2 may be less reliable surrogate markers for EKB-569 activity. In fact, one potential limitation when using pAKT or pERK1/2 as a surrogate marker is that these end points may overestimate the dose of EGF-R inhibitors needed for good antitumor activity.

The basis for the differential effect of EKB-569 on EGF-R and STAT3 *versus* AKT or ERK1/2 in cells stimulated with 10 or 100 ng/ml EGF is unknown. The level of pEGF-R, pAKT, pERK1/2, or pSTAT3-$Y^{705}$ stimulation by 10 or 100 ng/ml EGF is not markedly different and therefore does not account for the effect. Other *erb*B family members that undergo ligand-induced heterodimerization with EGF-R may be incompletely blocked by low levels of EKB-569 and may differentially participate in regulation of downstream pathways. A431 and NHEK cells express EGF-R, *erb*B-2, and *erb*B-3 so this possibility needs further consideration (33, 34). Alternatively, it may be that there are multiple phosphorylation sites on AKT and ERK1/2 that are differentially regulated by EGF compared with pEGF-R or pSTAT3. Because the phosphospecific antibodies detect only certain phosphorylation sites in these proteins, the antibodies may incompletely monitor total protein phosphorylation.

The specificity of inhibitory effect of EKB-569 on the EGF-R pathway and downstream signaling markers was examined by comparing the inhibitory effect of EKB-569 on the NF-κB pathway. In particular, we induced IKK-mediated degradation of IκBα in A431 cells with TNF-α. Stimulation of cells with a diverse array of stimuli, such as cytokines, TNF-α, interleukin-1, UV irradiation, and lipopolysaccharide, are known to result in phosphorylation of IκBα on both $Ser^{32}$ and $Ser^{36}$ (21). This results in the ubiquitination and degradation of IκBα, allowing NF-κB, a transcription factor, to translocate to the nucleus and activate transcription (26). It was shown that TNF-α induction caused degradation of IκBα, while EKB-569 did not have this effect. Although others have shown that EGF can induce NF-κB activation in an estrogen receptor-negative breast cancer cell line after 2 h (35), we believe that the absence of the effect of EKB-569 on IκBα in A431 cells indicates that the early effects of EKB-569 are specific on EGF-R, STAT3, AKT, or ERK1/2 and not IκBα. However, we cannot rule out effects on other pathways. In particular, while EKB-569 specifically inhibits EGF-R kinase compared with six other kinases (6), the effect of EKB-569 on the other >90 known protein tyrosine kinases or >500 kinase gene products in general (36) has not been explored.

To help translate our *in vitro* findings to a clinical setting, the effect of EKB-569 on downstream markers is being examined in A431 cells grown as tumors in nude mice. Previously, it was shown that 10–80 mg/kg EKB-569 given daily by oral gavage dramatically inhibited the growth of tumors derived from A431 cells (20) and inhibition of phosphorylation of EGF-R within the tumor is sustained for 24 h after oral administration at 10 mg/kg EKB-569 (6). In preliminary experiments, athymic nude mice bearing s.c. A431 tumors were given a single oral dose of 20 mg/kg EKB-569. One hour after administration of EKB-569, EGF-R and STAT3-$Y^{705}$ phosphorylation within the tumors was inhibited over 50%, confirming the association of the effect of EKB-569 on pEGF-R and pSTAT3-$Y^{705}$ observed *in vitro*. Further studies examining the relationship of surrogate protein markers as well as genomic profiling with EKB-569 exposure in animals will be presented in a future publication.

We conclude that the assessment of surrogate markers in human cancer, particularly STAT3 phosphorylation, should be further explored as a novel surrogate marker after therapeutic intervention with EGF-R inhibitors.

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