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Kahalalide F Induces Necrosis-Like Cell Death that Involves Depletion of ErbB3 and Inhibition of Akt Signaling

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ABSTRACT

Kahalalide F (KF) is a novel marine-derived antitumor agent that is currently undergoing phase II clinical trials. The mechanism of action of KF is not well understood. In line with previous reports, we show that KF caused rapid and potent cytotoxicity in the breast cancer cell lines SKBR3 and BT474, characterized by cytoplasmic swelling and DNA clumping. Several markers of caspase-dependent apoptosis, such as phosphatidyl-serine externalization, cytochrome *c* release, and caspase-3 and poly-(ADP-ribose) polymerase cleavage were negative after KF exposure. Inhibitors of caspases or cathepsins failed to protect against KF cytotoxicity. Altogether, these data indicate that KF-induced cell death is a necrosis-like process. The sensitivity to KF in a panel of human tumor cell lines derived from breast (SKBR3, BT474, and MCF7), vulval (A431), non–small-cell lung (H460, A549, SW1573, and H292), and hepatic (Skhep1, HepG2, and Hep3B) carcinomas positively correlated with ErbB3 (HER3) protein levels. A KF-resistant subline of colon carcinoma cells, HT29/KF, expressed significantly reduced levels of all ErbB receptors, but short-term KF exposure of sensitive cell lines such as SKBR3 selectively induced down-regulation of ErbB3. On the other hand, stable transfection of an ErbB3-expressing plasmid increased the KF sensitivity of H460 cells, the most resistant cell line in our panel. Finally, we found that KF efficiently inhibited the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway in sensitive cell lines and that ectopic expression of a constitutively active Akt mutant reduced KF cytotoxicity in this cell line. In summary, our results identify ErbB3 and the downstream PI3K-Akt pathway as important determinants of the cytotoxic activity of KF in vitro.

Kahalalide F (KF) is a novel antitumor agent that was originally isolated from the Hawaiian marine mollusk *Elysia rufescens* (Hamann and Scheuer, 1993; Hamann et al., 1996). KF has high cytotoxic activity against cell lines and tumor specimens derived from various human solid tumors, including prostate, breast, non–small-cell lung, ovarian, and colon carcinomas (Jimeno et al., 1996; Faircloth et al., 2000, 2001; Medina et al., 2001; Shao et al., 2001). Moreover, KF has shown antitumor activity against human prostate cancer xenografts in mouse models (Faircloth et al., 2000). In contrast, nontumoral cell lines were 5 to 40 times less sensitive to KF (Suarez et al., 2003), and bone marrow progenitors were not affected when treated with suprapharmacological concentrations of KF (Gomez et al., 2003). In a phase I clinical trial in solid tumors, antitumor activity was noted in patients harboring hepatoma, melanoma, and breast and pancreatic carcinoma (Ciruelos et al., 2002). The activity of KF is currently being investigated in phase II clinical trials in patients with melanoma, hepatic carcinoma, an NSCLC (Jimeno et al., 2004).

The molecular mechanism of action of KF is largely unknown. Lysosomes seem to be intracellular targets of KF, because human cervical tumor cells and monkey fibroblasts treated with KF formed large vacuoles and became dramatically swollen because of changes in lysosomal membranes (Garcia-Rocha et al., 1996). A recent report consistently demonstrated the loss of lysosomal integrity and the induction of severe cytoplasmic swelling and vacuolization in breast and

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ABBREVIATIONS: KF, kahalalide F; PARP, poly(ADP-ribose) polymerase; EGFR, epidermal growth factor receptor; NSCLC, non–small-cell lung cancer; Erk, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; GSK-3β, glycogen synthase kinase 3β; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; Ab, antibody; YFP, yellow fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PKB, protein kinase B; PI, propidium iodide; 7-AAD, 7-amino-actinomycin D; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; PS-341, *N*-((1*S*)-1-benzyl-2-(((1*R*)-1-(dihydroxyboranyl)-3-methylbutyl)amino)2-oxoethylpyrazinecarboxamide; CA-074, *N*-(3-propylcarbamoyloxirane-2-carbonyl)-isoleucyl-proline; zFA-fmk, *N*-benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone; zVAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

prostate cancer cells (Suarez et al., 2003). In the latter study, confocal laser and electron microscopy revealed that KF also induces damage of mitochondria, endoplasmatic reticulum, and the plasma membrane. In contrast, the nuclear membrane was preserved, and no DNA damage was detected, although the cell nucleus showed irregular clumping of chromatin. Ectopic overexpression of the multidrug resistance protein MDR1, inhibition of protein synthesis, or inhibition of caspase-dependent apoptosis did not significantly protect against KF cytotoxicity (Suarez et al., 2003).

Inhibition of ErbB signaling has been suggested to be part of the mechanism of KF action (Wosikowski et al., 1997; Faircloth et al., 2001), although ectopic overexpression of ErbB2 did not protect against KF-induced cell death (Suarez et al., 2003). Here we show that KF-induced cytotoxicity does not involve caspase-mediated apoptosis but is a necrosis-like cell death process. KF-induced cell death was also independent of the activity of the lysosomal proteases cathepsin B and D. The sensitivity to KF in a panel of cell lines derived from several tumor types, including breast, vulval, non–small-cell lung, and hepatic carcinoma, significantly correlated with protein expression levels of the ErbB3 receptor. We show that KF exposure induced down-regulation of ErbB3, whereas ectopic expression of ErbB3 increased the KF sensitivity of a resistant cell line. Finally, we found that KF efficiently inhibited the PI3K-Akt signaling pathway in sensitive cell lines and that ectopic expression of a constitutively active Akt mutant reduced KF cytotoxicity. Altogether, our data identify ErbB3 and Akt as major determinants of the cytotoxic activity of KF in vitro.

Materials and Methods

Chemicals. KF (1-oxa-4,7,10,13,16-pentaazacyclononadecane, cyclic peptide derivative) is manufactured by Pharmamar (Madrid, Spain) and was provided as a pure substance and diluted in dimethyl sulfoxide (DMSO)/ethanol (1:1; v/v). Stock solutions of zVAD-fmk (Enzyme Systems Products, Livermore, CA), calpeptin (Calbiochem, Darmstadt, Germany), CA-074 Me (Peptides International Inc., Osaka, Japan), zFA-fmk (Enzyme Systems Products), pepstatin A (Sigma Chemical, St. Louis, MO), and LY294002 (Cell Signaling Technology Inc., Beverly, MA) were made in DMSO.

Cell Culture and KF Selection. SKBR3, BT474, MCF7 (breast carcinoma), A431 (vulval carcinoma), NCI-H460 (H460), A549, SW1573, NCI-H292 (H292) (NSCLC), SKhep1, HepG2, Hep3B (hepatic carcinoma), and HT29 (colon carcinoma) cell lines were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium or RPM 1640 medium (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) supplemented with 10% (v/v) fetal calf serum (Invitrogen, Breda, The Netherlands), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells from exponentially growing cultures were used in all experiments. The KF-resistant HT29 cell line (HT29/KF) (supplied by Dr. Lola Garcia Grávalos, Pharmamar, Madrid, Spain) was obtained by treating parental HT29 cells with increasing concentrations of KF, from 0.15 to 1.3 μM KF, in which the cells grew well after 20 weeks of selection.

Flow Cytometry. Cells were plated at 40 to 60% confluence in 6- or 12-well plates. The following day, the medium was replaced with medium containing the drug(s) as indicated. Cells were treated for various times with 1 μM KF, unless otherwise indicated. Protease inhibitors were diluted in medium at final concentrations of 50 μM (zVAD-fmk), 100 μM (calpeptin, CA-074 Me, pepstatin A), or 200 μM (zFA-fmk), and added to the cells 1 h before the addition of KF. Cell-cycle analysis and apoptosis measurement were performed as described previously (Janmaat et al., 2003). In brief, the cell-cycle distribution was determined by propidium iodide (PI) staining of cells, which were resuspended in Nicoletti buffer (Nicoletti et al., 1991) and analyzed by flow cytometry. The extent of cell death was determined by the analysis of hypodiploid DNA using PI staining or by annexin V-fluorescein isothiocyanate and 7-amino-actinomycin D (7-AAD) double staining according to the manufacturer's protocol (Nexins Research, Kattendijk, The Netherlands). All analyses were performed on a fluorescence-activated cell-sorting calibur instrument using the CellQuest software package (BD Biosciences, San Jose, CA).

Western Blotting. Whole-cell lysates were denatured in sample buffer containing SDS and equal amounts of total protein, separated on 8 to 15% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk, the membranes were incubated overnight at 4°C with the first antibodies as indicated. The next day, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, and detection was performed using enhanced chemiluminescence reagent (Amersham Biosciences Inc., Piscataway, NJ). The antibodies used were anti-caspase-3, anti-phospho-EGFR (Tyr1068), anti-Erk, anti-phospho-Erk, anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-MDM2, anti-phospho-GSK-3β (all from Cell Signaling Technology), anti-PARP (Roche Diagnostics, Indianapolis, IN), anti-EGFR (Ab-12; Neomarkers Lab Vision Corporation, Fremont, CA), anti-c-ErbB2 (C18; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-c-ErbB2 (Tyr1248; Neomarkers Lab Vision Corporation), anti-c-ErbB3 (Ab-2; Neomarkers Lab Vision Corporation), and anti-c-ErbB-4 (Ab-2; Neomarkers). Protein expression levels were quantified using Bio-Rad software (Valencia, CA) and normalized with β-actin expression levels.

MTT Assays. Cells (5×10^3) were plated into flat-bottom 96-well plates (Costar, Corning, NY). After 24 h, various concentrations of KF were added, and the cells were incubated for an additional 72 h. Thereafter, 10% (v/v) of a solution of 5 mg/ml 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to each well and incubated for 3 h at 37°C. Plates were centrifuged for 5 min at 1200 rpm, and the medium was carefully discarded. The formed formazan crystals were dissolved in 100 μl of DMSO, and absorbance was determined at 540 nm using a Spectra Fluorimeter (Tecan, Salzburg, Austria). Absorbance values were expressed as the percentage of the untreated controls to calculate the concentration of KF resulting in 50% growth inhibition (IC_{50}).

Immunocytochemistry, Fluorescence Microscopy Analysis, and Quantification of Subcellular Distribution. Cells growing onto glass coverslips were fixed with 3.7% formaldehyde in PBS for 30 min, washed with PBS, permeabilized with 0.2% Triton in PBS for 10 min, and washed with PBS again. After a blocking step with 3% bovine serum albumin in PBS for 1 h, primary antibodies against $p27^{kip1}$ (BD Biosciences) or cytochrome *c* were diluted in blocking solution and applied for 1 h. After washing with PBS, cells were incubated with fluorescein isothiocyanate- (Sigma) or Alexafluor Red (Molecular Probes, Eugene, OR)-conjugated secondary antibodies for 1 h. Finally, the coverslips were rinsed three times with PBS and mounted onto microscope slides with Vectashield (Vector Laboratories, Burlingame, CA). The chromosome dye Hoechst 33285 (Sigma) was used to counterstain the nuclei. The immunostaining procedure was carried out at room temperature. Fluorescence microscopy analysis was carried out using an inverted Leica DMIRB/E fluorescence microscope (Leica Heidelberg, Heidelberg, Germany). Images were collected using the Q500MC Quantimet software V01.01 (Leica Cambridge, Cambridge, UK). To quantify the subcellular distribution of $p27^{kip1}$, the localization of the protein was determined in at least 200 cells per treatment.

Transfections. The mammalian expression vectors pBABE-ErbB3 (Holbro et al., 2003) and pSG5-gag-PKB (Burgering and Coffer, 1995) were generously provided by Dr. N. Hynes (Friedrich Miescher Institute, Basel, Switzerland) and Dr. P. Coffer (University Medical Center, Utrecht, The Netherlands), respectively. Cells were

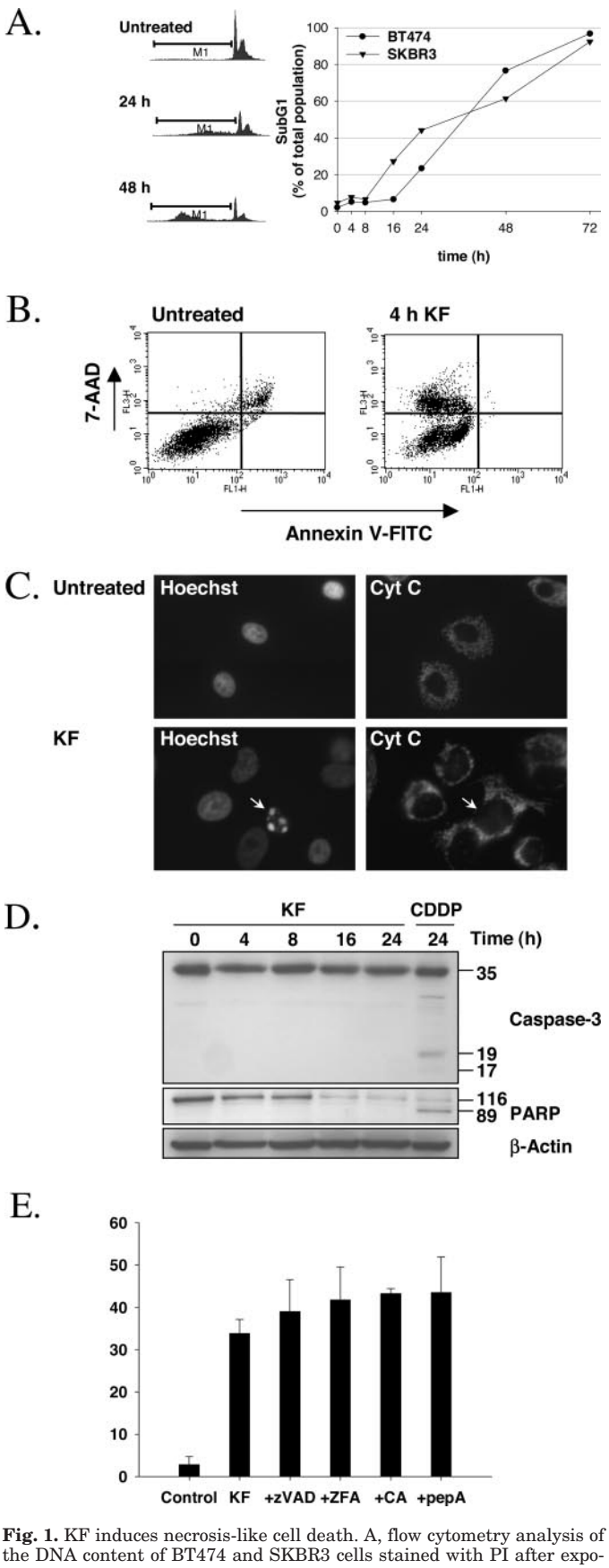

Fig. 1. KF induces necrosis-like cell death. A, flow cytometry analysis of the DNA content of BT474 and SKBR3 cells stained with PI after exposure to KF (1 μM) for different times. Representative examples of the cell-cycle distribution of SKBR3 cells untreated or treated for 24 or 48 h with KF are shown (left). The number of cells with hypodiploid DNA content (M1) were measured and presented as the percentage of total cell population (right). B, KF-treated SKBR3 cells (1 μM for 4 h) were double-stained with annexin-V/7-AAD and analyzed by flow cytometry. Gate settings distinguish between living (bottom left), necrotic (top left), early apoptotic (bottom right), and late apoptotic (top right) cells. C, cytochrome *c* is not released from mitochondria in cells affected by KF treatment. Representative examples of SKBR3 cells stained for Hoechst (left) and cytochrome *c* (Cyt C) (right) incubated without (untreated) or with KF (1 μM) for 2 h. The arrows at the bottom indicate a cell with clumped DNA but no cytochrome *c* release. D, effect of KF on caspase-3 and PARP cleavage. SKBR3 cells were incubated for the indicated period of time with KF, and effects on caspase-3 and PARP were analyzed by Western blotting. As a control, cells were treated with cisplatin (10 μM) for 24 h (lane 6). The membranes were stripped and reprobed with anti-β-actin to verify equal protein loading. E, no effect of caspase or cathepsin B or D inhibition on KF-induced sub-G_1 population. SKBR3 cells were pretreated with the pan-caspase inhibitor zVAD-fmk (50 μM), the cathepsin B inhibitors zFA-fmk (ZFA) or CA-074 (CA), or the cathepsin D inhibitor pepstatin A (pepA) for 1 h before the addition of KF (1 μM). The sub-G_1 population was measured after 24 h as in A. Values represent the mean and S.D. of three independent experiments.

seeded in 6- or 12-well trays and transfected with 0.5 to 2 μg of plasmid DNA using the FuGene6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's protocol. For transient transfection experiments, cells were transfected with the YFP-expression plasmid pEYFP-C1 (BD Biosciences Clontech, Palo Alto, CA) alone or together with the pSG5-gag-PKB vector. Using YFP expression as a marker of transfection, we determined the percentage of transfected cells that detached after treatment with KF. The expression of gag-PKB was confirmed by Western blotting in a parallel sample. Finally, to make stable ErbB3-expressing cells, H460 cells were transfected with 5 to 10 μg of pBABE-ErbB3 cDNA or empty vector control vectors using Superfect reagent (Invitrogen) according to the manufacturer's protocol. Transfected cells were selected in puromycin-containing medium, and the pooled population was used.

Statistics. Correlation coefficients (r) and accompanying P values were calculated using SPSS software (SPSS Inc., Chicago, IL). Quantitative experiments were analyzed by the Student's t test, and P values resulted from the use of two-sided tests and were considered significant when ≤ 0.05.

Results

KF-Induced Cytotoxicity Is Mediated through a Necrosis-Like Cell-Death Process. To investigate whether KF induces the activation of caspase-dependent apoptosis, we examined several apoptotic markers after treatment with KF. First, cell-cycle analysis revealed that KF induces a hypodiploid cell population in SKBR3 and BT474 cells starting after 16- to 24-h exposure to KF and increasing to almost 100% after 72 h (Fig. 1A). Flow cytometric analysis after 7-AAD/Annexin-V double staining demonstrated that KF caused a rapid disruption of the cell membrane, as visualized by the increase of 7-AAD staining (Fig. 1B). However, Annexin-V staining remained negative after treatment (Fig. 1B), indicating that KF does not induce the externalization of phosphatidyl-serine, which is characteristic of apoptotic cell death. KF treatment resulted in DNA clumping, but cytochrome *c* remained within mitochondria even in cells clearly affected by KF treatment (Fig. 1C, indicated by the arrow). The sensitivity to KF of A549 cells overexpressing the anti-apoptotic protein Bcl-2 was similar to that of nontransfected cells (data not shown), further indicating that apoptosis was not involved in the action of KF. Neither caspase-3 nor PARP was cleaved after up to 24-h exposure to KF (Fig. 1D, top), and cotreatment with the broad caspase inhibitor zVAD-fmk

did not prevent the hypodiploid cell population induced by KF (Fig. 1E). PARP was degraded after 16-h exposure to KF, but the 89-kDa caspase-dependent cleavage product that appeared in control cells treated with cisplatin was not observed (Fig. 1D, middle). This suggests that other proteases were involved in KF-induced degradation of PARP.

Previous reports that KF induces the loss of lysosomal integrity (Garcia-Rocha et al., 1996; Suarez et al., 2003) and that PARP can be degraded by lysosomal cathepsins during necrosis (Gobeil et al., 2001) raised the possibility that the observed KF-induced PARP degradation could be mediated by these proteases and that cathepsins might be involved in the action of KF. To investigate this possibility, cells were cotreated with zFA-fmk or CA-074-ME, which are specific inhibitors of cathepsin B, or with the cathepsin D inhibitor pepstatin A. As shown in Fig. 1E, inhibition of cathepsin B or D failed to protect from the KF-induced sub-G_1 population, demonstrating that the activity of cathepsin B or D is not required for KF-mediated cytotoxicity. Taken together, these data demonstrate that KF does not activate caspase-dependent apoptosis but causes cell death that resembles necrosis.

ErbB3 Expression Levels Correlate with KF Sensitivity. Using normal human skin fibroblasts, we confirmed previous reports (Gomez et al., 2003; Suarez et al., 2003) that

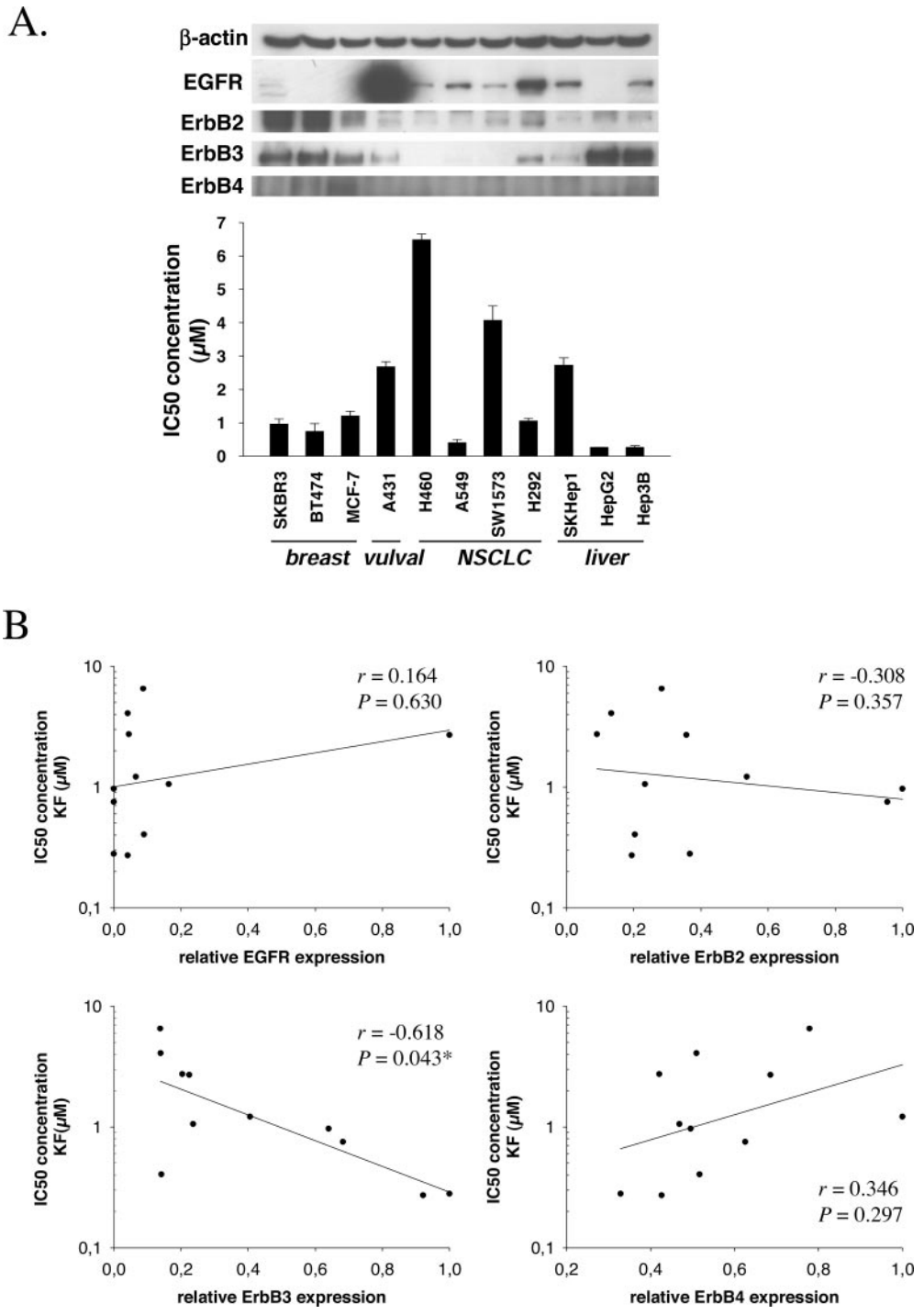

Fig. 2. ErbB3 protein expression levels correlate with KF sensitivity. Correlation of KF sensitivity with expression of the ErbB family of kinases. A, basal ErbB expression levels were evaluated by western blotting. IC_{50} values of KF represent the mean and S.D. of at least three independent MTT assays (see *Materials and Methods*). B, the ErbB protein expression levels shown in A were quantified using Quantity One (Bio-Rad) software, normalized with β-actin expression levels, and plotted against the mean IC_{50} values of KF. The *r* and *P* values were calculated using SPSS software.

nontumoral cells have 5 to 40 times higher IC_{50} values for KF compared with sensitive tumor cells (data not shown). Malignant cells commonly possess overactivated signal-transduction cascades that provide potential selective targets of antitumor drugs (Blume-Jensen and Hunter, 2001). Because KF inhibits the EGFR and ErbB2 receptors and down-regulates transforming growth factor-α gene expression (Wosikowski et al., 1997; Faircloth et al., 2001), and high expression of the ErbB family of receptor tyrosine kinases is prevalent in tumor cells (Salomon et al., 1995), we investigated whether protein expression levels of ErbB receptors (ErbB1–4) correlate with the sensitivity to KF in a panel of tumor cell lines with variable expression of each receptor. The IC_{50} values for KF, as measured in MTT assays, ranged from 0.1 to 7 μM within the panel of cell lines (Fig. 2A). The sensitivity of the cell lines to KF did not correlate with their EGFR (ErbB1), ErbB2, or ErbB4 protein expression levels (Fig. 2B). However, ErbB3 levels showed a significant inverse correlation ($r = -0.61$, $P = 0.0428$) with IC_{50} values of KF (Fig. 2B).

KF Induces Down-Regulation of ErbB3 Protein. Western blot analysis was carried out to investigate potential differences in the expression levels of ErbB receptors in the KF-sensitive HT29 colon carcinoma cell line compared with a KF-resistant subline (HT29/KF) generated by long-term exposure to increasing concentrations of the drug, and it was capable of growing in the presence of 1.3 μM KF (see *Materials and Methods*). The expression of all four ErbB receptors was down-regulated in the KF-resistant subline compared with the parental cell line (Fig. 3A). In contrast to the general down-regulation of ErbB family members observed after long-term exposure to KF, a 4-h treatment with KF resulted in the selective down-regulation of ErbB3 in sensitive, high ErbB3-expressing SKBR3 cells (Fig. 3B).

Ectopic ErbB3 Expression Increases the Sensitivity of H460 Cells to KF. To investigate whether ectopic ErbB3 expression affects the sensitivity of low ErbB3-expressing cells to KF treatment, H460 cells were transiently cotransfected with cDNA, encoding ErbB3 (Holbro et al., 2003) and YFP as a marker of transfection. In comparison to control cells cotransfected with an empty vector and YFP, an increased percentage of YFP-positive, detached cells was noted in ErbB3 cotransfected cells upon KF treatment (data not shown). Next, H460 cells were stably transfected with an ErbB3-encoding cDNA. These cells showed increases of bands of approximately 125 and 30 kDa, detected with an ErbB3-specific antibody, whereas no induction of full-length ErbB3 was observed (Fig. 4A, the different isoforms are indicated by arrows). Treatment of the H460-pBABE-ErbB3 cells with 2 μM KF resulted in decreased expression of the ErbB3-related 125- and 30-kDa proteins (Fig. 4A), similar to previously demonstrated KF-mediated depletion of full-length ErbB3. In line with the results in transient transfected cells, a higher fraction of the H460-pBABE-ErbB3 cell line exhibited morphological signs of KF-induced cell death compared with control cells transfected with an empty vector (Fig. 4B). Moreover, H460-pBABE-ErbB3 cells showed a modest but significant ($p = 0.026$) reduction of the IC_{50} concentration of KF compared with empty vector-transfected cells (Fig. 4C). Altogether, the results suggest that ectopic ErbB3 expression sensitizes these cells for KF treatment.

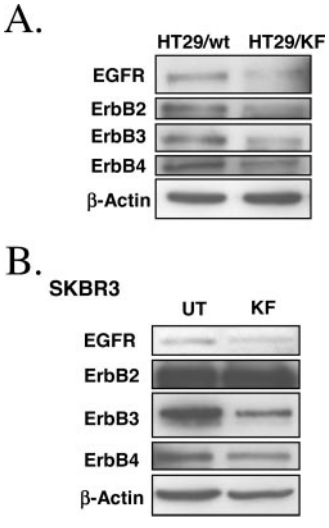

Fig. 3. KF induces down-regulation of ErbB3 protein. Western blot analysis of protein expression levels of ErbB receptors in HT29 wild-type (HT29/wt) and KF-resistant HT29 (HT29/KF) cells that were exposed to KF for a long period of time (A) and in SKBR3 cells treated with KF (1 μM) for a short period of time (4 h) (B). The membranes were stripped and reprobed with anti-β-actin to verify equal protein loading.

KF Depletes ErbB3 and Inhibits Akt in KF-Sensitive Cells. When a panel of other tumor cell lines was analyzed, we observed that KF induced ErbB3 down-regulation specifically in sensitive cell lines (Fig. 5A). All ErbB receptors couple to two major signaling cascades, the mitogen-activated protein kinase pathway involving Ras/Erk, and the PI3K/Akt cascade (Yarden and Sliwkowski, 2001), but ErbB3 is the most efficient activator of PI3K (Prigent and Gullick, 1994). Both kinase pathways are involved in the regulation of cell proliferation and survival and are often overactivated in tumor cells (Blume-Jensen and Hunter, 2001). Phosphorylated or total levels of Erk were not affected by exposure of the sensitive SKBR3 cells to KF for up to 24 h (Fig. 5B). In contrast, phosphorylated but not total Akt levels decreased within only 30 min after the addition of KF to SKBR3 cells with the maximal effect reached after 2 h, which was sustained for up to at least 24 h (Fig. 5B). When the complete panel of cell lines was analyzed, we found a decrease of Akt phosphorylation only in those cell lines in which KF provoked down-regulation of ErbB3 (Fig. 5A), suggesting that KF-mediated Akt inhibition was caused by ErbB3 depletion. In contrast, like ErbB3 expression, Akt phosphorylation remained largely unaffected in the KF-resistant cell lines, including the acquired resistant subline HT29/KF (Fig. 5A). It is interesting that the basal levels of phosphorylated Akt were increased in the HT29/KF cell line compared with its parental cell line (Fig. 5A).

In a titration experiment in SKBR3 cells, the decrease of phosphorylated Akt started at concentrations of 0.25 μM, having the largest effect at 1 μM (Fig. 5C). Consistent with the inhibition of Akt activity, the phosphorylation of the Akt substrates MDM2 as well as GSK-3β decreased in cells exposed to KF (Fig. 5C). As an additional marker for Akt activity, the nuclear/cytoplasmic localization of $p27^{kip1}$ was evaluated by immunofluorescence. Nuclear $p27^{kip1}$ inhibits cyclin/cyclin-dependent kinase complexes resulting in an arrest of cells in the G_1 phase of the cell cycle (Polyak et al., 1994; Toyoshima and Hunter, 1994). Phosphorylation of

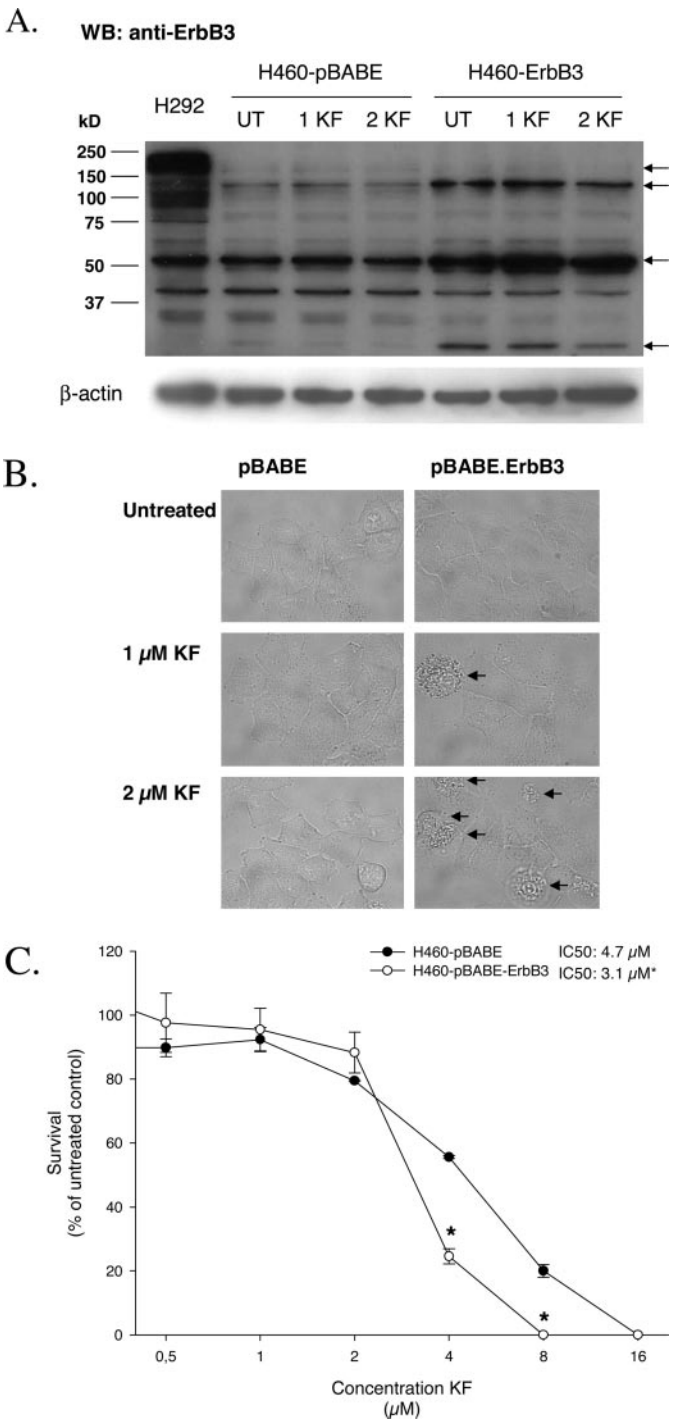

Fig. 4. Ectopic ErbB3 expression increases the sensitivity of H460 cells to KF. A, ErbB3 protein expression was analyzed by Western blotting on total cell lysates of H460 cells that were stably transfected with the empty pBABE vector or the pBABE-ErbB3 vector. Effect of KF treatment on ErbB3 expression was analyzed on cells treated with 1 or 2 μM KF for 4 h. A total cell lysate of H292 cells was included as a positive control. The arrows indicate the full-length ErbB3 of 185 kDa and truncated ErbB3 isoforms of approximately 125, 60, and 30 kDa. Note the decrease of these proteins after KF treatment. B, phase contrast pictures of H460 cells stably expressing the empty pBABE vector or the pBABE-ErbB3 vector, exposed to 0, 1, or 2 μM KF for 4 h. The arrows indicate cells that were visually affected by KF treatment. C, H460.ErbB3 cells are more sensitive for KF treatment than H460.pBABE cells. Growth inhibition induced by various concentrations of KF was determined in MTT assays at 72 h. The percentage of survival was expressed as the percentage of the untreated controls. Mean, S.D., and IC_{50} values are shown from two independent experiments ($\star$, $P \leq 0.05$, empty vector versus ErbB3, Student's t test).

p27^{kip1} by Akt results in the confinement of p27^{kip1} in the cytoplasm, thus stimulating cell-cycle progression (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002). Treatment with KF induced redistribution of p27^{kip1} from the cytosol to the nucleus to an extent similar to that of the selective PI3K inhibitor LY294002, included as a control, further demonstrating that Akt activity was inhibited (Fig. 5D).

Cells treated with KF exhibited extensive cytoplasmic swelling and rapidly detached from the bottom of the tissue-culture tray. After treatment with KF for 1 h, detached cells were separately harvested from attached cells. Down-regulation of ErbB3 and inhibition of Akt was only observed in detached cells (Fig. 5E), indicating that these events coincide with KF-induced cytotoxicity.

Constitutive Activation of Akt Protects SKBR3 Cells from KF-Induced Cytotoxicity. KF-mediated Akt dephosphorylation requires the activity of protein phosphatases PP2A and/or PP1, because pretreatment with the phosphatase inhibitor okadaic acid prevented KF-induced Akt dephosphorylation (data not shown). However, okadaic acid failed to protect against KF-induced cytotoxicity (data not shown), probably because of additional toxic effects of PP1 and PP2A inhibition. Hence, as another approach to prevent KF-induced inactivation of Akt, cells were transfected with a plasmid encoding for gag-PKB, a constitutively active mutant of Akt (Burgering and Coffer, 1995). A smaller number of cells that were transiently cotransfected with gag-PKB and YFP exhibited morphological signs of KF-induced cell death compared with cells transfected with YFP alone (Fig. 6A). As shown in Fig. 6B, cotransfection with gag-PKB significantly reduced the percentage of YFP-positive detached cells upon KF treatment, indicating that constitutively active Akt protects against KF cytotoxicity.

Discussion

In the study presented here, we investigated the molecular mechanism of action of the novel marine antI-cancer compound KF, which is currently being investigated in phase II clinical trials. In line with other studies (Garcia-Rocha et al., 1996; Suarez et al., 2003), we observed rapid and potent cytotoxic activity against ErbB2-overexpressing breast cancer cell lines, which was associated with the induction of a hypodiploid cell population, dramatic cytoplasmic swelling, and permeabilization of the plasma and lysosomal membranes. The cell death induced by KF cannot be classified as apoptosis, because several apoptotic markers were negative after KF exposure, and molecular and chemical inhibitors of caspase-dependent cell death failed to protect against KF. In addition to caspase-mediated apoptosis, cells can activate alternative types of programmed cell death mediated by other proteases, such as calpains and cathepsins (Leist and Jaättela, 2001). However, specific inhibitors of the lysosomal proteases cathepsin B or D (Fig. 1E) or of the protease calpain (data not shown) also failed to protect against KF-induced cytotoxicity. In line with a previous study in prostate and breast cancer cells (Suarez et al., 2003), our data indicate that KF induces a necrosis-like cell death process.

We provide several lines of evidence that point to ErbB3 as a major determinant of KF action. First, the inverse correlation between ErbB3 expression and KF IC_{50} values within the panel of human tumor cell lines suggests ErbB3 as a

marker for KF sensitivity. Second, exposure to KF results in down-regulation of ErbB3 protein expression, which was observed in cells exposed for a short time (4 h) to KF and in KF-resistant cells that were selected over a longer period of time in KF-containing medium. Third, KF treatment induced down-regulation of ErbB3 primarily in the detached, dying cell fraction but not in the attached cell fraction. Finally, H460 cells ectopically expressing ErbB3 were more sensitive for KF treatment than were cells transfected with an empty vector. Together, this indicates that down-regulation of ErbB3 in cells that depend on ErbB3 for their survival contributes to the cytotoxicity of KF.

It is interesting that H460 cells transfected with full-length ErbB3 cDNA expressed a smaller, 125-kDa protein. Others have demonstrated that ErbB3 exists in several isoforms of different sizes, including the truncated extracellular domain of approximately 30 kDa in size, which is located as a 58-kDa disulphide-linked dimer in vesicles in the cytoplasm (Lee and Maihle, 1998; Srinivasan et al., 2001). However, the 125-kDa protein that we also observed has not been described. Underglycosylation or proteolytic cleavage of the full-length protein may potentially account for the reduced ErbB3 protein size in H460 cells. In turn, low expression levels of full-length, functional ErbB3 is one of the factors

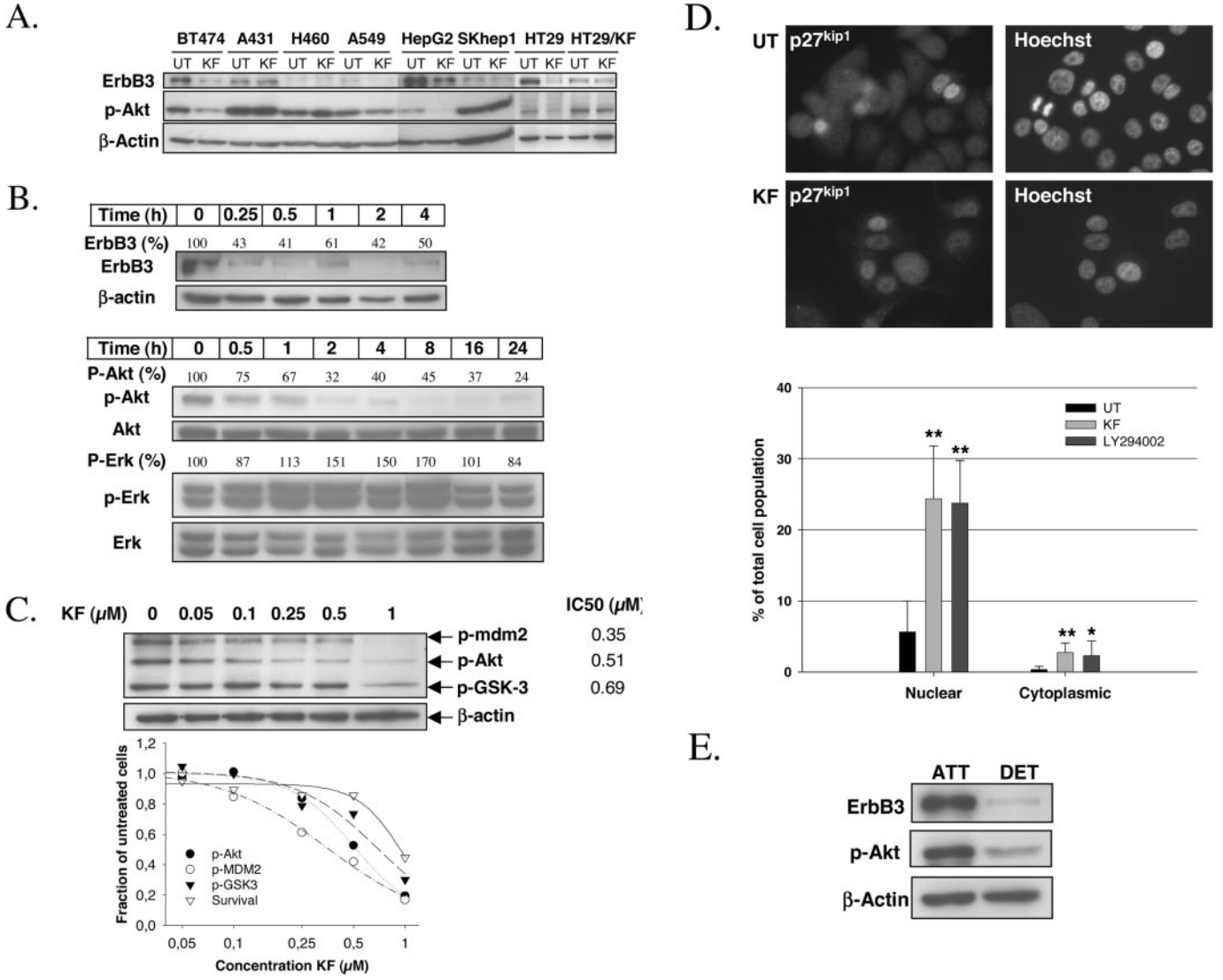

Fig. 5. KF depletes ErbB3 and inhibits Akt in sensitive cells. A, the indicated cell lines were left untreated (UT) or exposed to KF (1 μM) for 4 h, and ErbB3 and phosphorylated (p)-Akt expression levels were analyzed by Western blotting. The membrane was stripped and reprobed with anti-β-actin to verify equal protein loading. B, SKBR3 cells were exposed to KF (1 μM) for different times, and ErbB3 and total and p-Erk and Akt were analyzed by Western blotting. Protein expression levels of ErbB3, p-Akt, and p-Erk were quantified, normalized with β-actin, and calculated as percentage of untreated cells. C, SKBR3 cells were exposed to different concentrations of KF as indicated, and p-Akt, mdm2, and GSK-3β were analyzed by Western blotting. The IC_{50} values for KF were calculated after quantification of the Western blots. The lower panel shows the quantified and normalized protein expression levels of p-Akt, p-MDM2, and p-GSK3β plotted against the KF concentration. The graph also shows the survival of SKBR3 cells after 72-h treatment plotted against the KF concentration as measured in MTT assays (see *Materials and Methods*). D, representative examples of SKBR3 cells incubated without (UT) or with KF (1 μM) for 4 h and stained for $p27^{kip1}$ and Hoechst. The proportion of cells showing predominantly nuclear localization of $p27^{kip1}$ or predominant cytoplasmic $p27^{kip1}$ localization is shown at the bottom, including cells that were treated with the PI3K inhibitor LY294002, used as a control. The proportion of cells that had both nuclear and cytoplasmic $p27^{kip1}$ localization is not shown. Values represent the mean and S.D. by counting at least 200 cells/treatment in two independent experiments ($\star$, $P \leq 0.05$; $\star\star$, $P \leq 0.01$, untreated versus treated, Student's t test). E, SKBR3 cells were exposed for 1 h with KF (1 μM), and the attached and detached cell populations were separately harvested and analyzed for ErbB3 and phosphorylated Akt protein levels.

that may account for the limited effect of ErbB3 transfection. Several other factors, however, might contribute to the relatively small differences between the IC_{50} values of ErbB3- and empty vector-transfected H460 cells. On one hand, because ErbB3 lacks intrinsic kinase activity, functional ErbB3 also requires the expression of other ErbB receptors, which is poor in H460 cells (Fig. 2). On the other hand, the use of pooled transfectants may have masked larger cytotoxic effects induced by KF, because a subpopulation of the cells may express only small amounts of ErbB3.

KF-mediated depletion of ErbB3 was accompanied by a rapid decrease in Akt phosphorylation and inhibition of downstream signaling, an effect that was only observed in sensitive, ErbB3-expressing cells. This finding, together with our observation that blockage of the PI3K/Akt pathway with the PI3K inhibitor LY294002 did not affect ErbB3 expression (data not shown), suggests that inhibition of Akt signaling reflects KF-mediated ErbB3 depletion. This is also consistent with the notion that functional ErbB3 is required for Akt activity in cells that depend on ErbB2 and ErbB3 for their growth and survival, such as SKBR3 cells (Holbro et al., 2003).

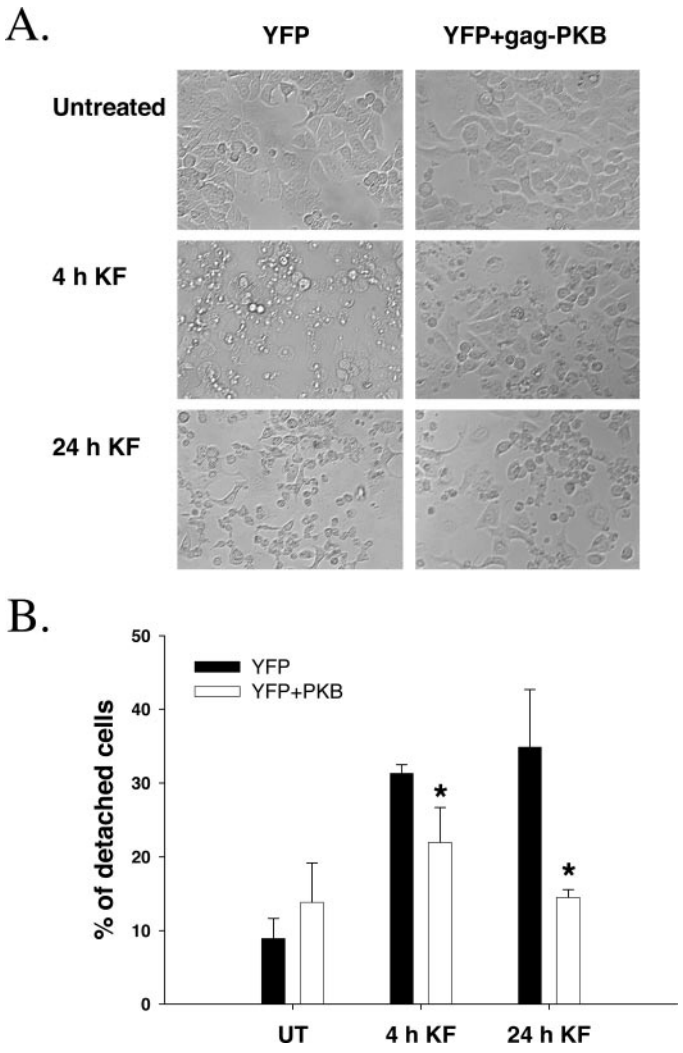

Fig. 6. Constitutive activation of Akt protects SKBR3 cells from KF-induced cytotoxicity. A, phase contrast pictures of cells transfected with YFP alone (YFP) or YFP and gag-PKB (YFP + gag-PKB), which were left untreated or exposed to KF (1 μM) for 4 or 24 h. B, fraction of YFP-positive cells that had detached from the bottom of the tissue culture tray after exposure to KF (1 μM) for 4 or 24 h. Mean and S.D. are shown from two independent assays by counting at least 300 cells/treatment ($\star$, $P \leq 0.05$, YFP versus YFP + gag-PKB, Student's t test).

Our data indicate that KF-mediated inhibition of the ErbB3-Akt pathway contributes to KF cytotoxicity, because cells transfected with a constitutively active mutant of Akt were largely protected against KF cytotoxicity. Moreover, the KF-resistant cell line HT29/KF showed increased basal Akt phosphorylation compared with its parental, KF-sensitive cell line HT29, which was not decreased after KF exposure. However, no KF-mediated inhibition of Akt phosphorylation was observed in ErbB3-transfected H460 cells (data not shown). Akt activity is partially uncoupled from growth factor receptor activity in H460 cells (Janmaat et al., 2003), providing a possible explanation for the observation that ErbB3 depletion does not affect Akt activity in these cells. Akt is a major downstream target of receptor tyrosine kinases that signal via PI3K (Burgering and Coffer, 1995) and possesses prosurvival and antiapoptotic activities (Franke et al., 2003). Although the role of Akt in apoptosis suppression is well established (Franke et al., 2003), little is known about the involvement of Akt in necrosis-like cell death. Akt inactivation has been observed in multiple types of caspase-independent cell deaths induced by agents such as *N*-methyl-D-aspartate, nitric oxide, hydrogen peroxidase, and ansamycin antibiotics (Basso et al., 2002; Luo et al., 2003). Similar to our results with KF, the introduction of a constitutively active mutant of Akt suppressed *N*-methyl-D-aspartate toxicity (Luo et al., 2003). However, the exact underlying mechanism remains to be investigated.

The demonstration that ErbB3 expression levels correlated with KF sensitivity may explain the reported preferential effect of KF on tumor versus normal cells (Gomez et al., 2003; Suarez et al., 2003), because ErbB3 is commonly overexpressed in tumors (Salomon et al., 1995). However, the NSCLC cell line A549, with low ErbB3 levels, was highly sensitive for KF, suggesting that ErbB3 expression levels alone are not predictive of KF sensitivity in all cases. Although A549 cells show low ErbB3 expression, their survival is dependent on ErbB3 expression, because selective ErbB3 depletion using RNA interference has been shown to induce cell death in this cell line (Sithanandam et al., 2004).

In contrast to an earlier observation of KF-mediated inhibition of EGFR and ErbB2 (Wosikowski et al., 1997; Faircloth et al., 2001), we did not detect any significant, direct effect of KF treatment on EGFR or ErbB2 expression (Fig. 3) or phosphorylation in A431 or SKBR3 cells, respectively (data not shown). However, the observation that besides ErbB3, other ErbB receptors were also down-regulated to some extent during the selection process of the KF-resistant HT29 subline might reflect an indirect effect of KF on the expression of other ErbB receptors and suggests a potential role of multiple members of the ErbB family in KF sensitivity.

The mechanism by which ErbB3 is down-regulated by KF remains to be clarified. KF-induced down-regulation of ErbB3 is not caused by inhibited synthesis of the receptor, because treatment of SKBR3 cells with the protein synthesis inhibitor cyclohexamide (50 μM) for 4 h did not affect ErbB3 expression levels, whereas expression of $p27^{kip1}$ was down-regulated in these cells (data not shown). These data thus indicate that ErbB3 down-regulation is caused by degrada-

tion rather than inhibition of protein synthesis. ErbB3 protein can be ubiquitinated by the ubiquitin ligase Nrpd1 and subsequently degraded by proteasomes (Diamonti et al., 2002; Qiu and Goldberg, 2002). However, KF-induced ErbB3-degradation was not proteasome-mediated, because cotreatment of cells with the proteasome inhibitors MG-132 or PS-341 failed to protect from KF-induced ErbB3 depletion (data not shown). On the other hand, KF might mediate the internalization of ErbB3 and subsequently target it for degradation, similar to epidermal growth factor-mediated internalization of the EGFR (French et al., 1995). However, it remains to be elucidated if and how KF binds ErbB3.

The data presented here may have important clinical relevance. We demonstrate that KF is active in vitro against cells derived from various tumor types, including breast, colon, NSCLC, and hepatic carcinomas at clinically relevant concentrations. Moreover, our finding that ErbB3 expression levels correlate with sensitivity of cell lines to KF suggests ErbB3 as a marker for KF sensitivity. Furthermore, downregulation of ErbB3 expression or inhibition of Akt or downstream events, such as the cellular localization of $p27^{kip1}$, could serve as surrogate markers for KF activity. Although these preclinical findings require confirmation in the clinic, our data further suggest that mutations leading to constitutive activation of the PI3K/Akt pathway, such as depletion of *PTEN* (Stambolic et al., 1998) or *PIK3CA* mutations (Samuels et al., 2004) that have been identified in several tumor types, may contribute to mechanisms of resistance to KF.

References

Basso AD, Solit DB, Munster PN, and Rosen N (2002) Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. *Oncogene* **21:**1159–1166.

Blume-Jensen P and Hunter T (2001) Oncogenic kinase signalling. *Nature (Lond)* **411:**355–365.

Burgering BM and Coffer PJ (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature (Lond)* **376:**599–602.

Ciruelos C, Trigo T, Pardo J, Paz-Ares L, Estaun N, Cuadra C, Domínguez M, Marín A, Jimeno JM, and Izquierdo M (2002) A phase I clinical and pharmokinetic (PK) study with kahalalide F (KF) in patients (Pts) with advanced solid tumors (AST) with a continuous weekly (W) 1-hour IV infusion schedule. *Eur J Cancer* **38 (Suppl 7):**S33.

Diamonti AJ, Guy PM, Ivanof C, Wong K, Sweeney C, and Carraway KL III (2002) An RBCC protein implicated in maintenance of steady-state neuregulin receptor levels. *Proc Natl Acad Sci USA* **99:**2866–2871.

Faircloth GT, Grant W, Smith B, Supko J, Brown A, Geldof A, and Jimeno JM (2000) Preclinical development of kahalalide F, a new marine compound selected for clinical studies. *Proc Am Assoc Cancer Res* **41:**600.

Faircloth GT, Smith B, Grant W, Jimeno JM, García-Grávalos L, Scotto K, and Shtil A (2001) Selective antitumor activity of kahalalide F, a marine-derived cyclic depsipeptide. *Proc Am Assoc Cancer Res* **42:**213.

Franke TF, Hornik CP, Segev L, Shostak GA, and Sugimoto C (2003) PI3K/Akt and apoptosis: size matters. *Oncogene* **22:**8983–8998.

French AR, Tadaki DK, Niyogi SK, and Lauffenburger DA (1995) Intracellular trafficking of epidermal growth factor family ligands is directly influenced by the pH sensitivity of the receptor/ligand interaction. *J Biol Chem* **270:**4334–4340.

Garcia-Rocha M, Bonay P, and Avila J (1996) The antitumoral compound kahalalide facts on cell lysosomes. *Cancer Lett* **99:**43–50.

Gobeil S, Boucher CC, Nadeau D, and Poirier GG (2001) Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases. *Cell Death Diff* **8:**588–594.

Gomez SG, Bueren JA, Faircloth GT, Jimeno J, and Albella B (2003) In vitro toxicity of three new antitumoral drugs (trabectedin, aplidin and kahalalide F) on hematopoietic progenitors and stem cells. *Exp Hematol* **31:**1104–1111.

Hamann MT, Otto CS, Scheuer PJ, and Dunbar DC (1996) Kahalalides: bioactive peptides from a marine mollusk elysia rufescens and its algal diet *Bryopsis* sp. (1). *J Org Chem* **61:**6594–6600.

Hamann MT and Scheuer PJ (1993) Kahalalide F: a bioactive depsipeptide from the sacoglossan mollusk *Elysia rufescens* and the green alga *Bryopsis* sp. *J Am Chem Soc* **115:**5825–5826.

Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF III, and Hynes NE (2003) The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci USA* **100:**8933–8938.

Janmaat ML, Kruyt FA, Rodriguez JA, and Giaccone G (2003) Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. *Clin Cancer Res* **9:**2316–2326.

Jimeno J, Lopez-Martin JA, Ruiz-Casado A, Izquierdo MA, Scheuer PJ, and Rinehart K (2004) Progress in the clinical development of new marine-derived anticancer compounds. *Anticancer Drugs* **15:**321–329.

Jimeno JM, Faircloth GT, Cameron L, Meely K, Vega E, Gómez A, Fernández Sousa-Faro JM, and Rinehart K (1996) Progress in the acquisition of new marine-derived anticancer compounds: development of ecteinascidin-743 (ET-743). *Drugs Future* **21:**1155–1165.

Lee H and Maihle NJ (1998) Isolation and characterization of four alternate C-ErbB3 transcripts expressed in ovarian carcinoma-derived cell lines and normal human tissues. *Oncogene* **16:**3243–3252.

Leist M and Jaättela M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* **2:**589–598.

Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, et al. (2002) PKB/Akt phosphorylates P27, impairs nuclear import of P27 and opposes P27-mediated G1 arrest. *Nat Med* **8:**1153–1160.

Luo HR, Hattori H, Hossain MA, Hester L, Huang Y, Lee-Kwon W, Donowitz M, Nagata E, and Snyder SH (2003) Akt as a mediator of cell death. *Proc Natl Acad Sci USA* **100:**11712–11717.

Medina LA, Gómez L, Cerna C, Faircloth GT, Yochmowitz M, and Weitman S (2001) Investigation of the effects of kahalalide F (PM92102) against tumor specimens taken directly from patients. *Proc Am Assoc Cancer Res* **42:**213.

Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, and Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139:**271–279.

Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, and Massague J (1994) Cloning of P27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78:**59–66.

Prigent SA and Gullick WJ (1994) Identification of C-ErbB-3 binding sites for phosphatidylinositol 3′-kinase and SHC using an EGF receptor/c-ErbB-3 chimera. *EMBO (Eur Mol Biol Organ) J* **13:**2831–2841.

Qiu XB and Goldberg AL (2002) Nrdp1/FLRF is a ubiquitin ligase promoting ubiquitination and degradation of the epidermal growth factor receptor family member, ErbB3. *Proc Natl Acad Sci USA* **99:**14843–14848.

Salomon DS, Brandt R, Ciardiello F, and Normanno N (1995) Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* **19:**183–232.

Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, et al. (2004) High Frequency of Mutations of the PIK3CA gene in human cancers. *Science (Wash DC)* **304:**554.

Shao L, Weissbach L, Faircloth GT, Chabner BA, and Hornicek FJ (2001) In vitro anti-proliferative effect on sarcoma cells of ET-743 and other marine chemotherapeutics. *Proc Am Assoc Cancer Res* **42:**203.

Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J, and Arteaga CL (2002) PKB/Akt mediates cell-cycle progression by phosphorylation of P27(Kip1) at threonine 157 and modulation of its cellular localization. *Nat Med* **8:**1145–1152.

Sithanandam G, Fornwald L, Fields J, and Anderson L (2004) Inactivation of ErbB3 by siRNA blocks growth and promotes apoptosis in human lung adenocarcinoma cell line A549. *Proc Am Assoc Cancer Res* **45:**2426.

Srinivasan R, Leverton KE, Sheldon H, Hurst HC, Sarraf C, and Gullick WJ (2001) Intracellular expression of the truncated extracellular domain of C-ErbB-3/HER3. *Cell Signal* **13:**321–330.

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, and Mak TW (1998) Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95:**29–39.

Suarez Y, Gonzalez L, Cuadrado A, Berciano M, Lafarga M, and Munoz A (2003) Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2:**863–872.

Toyoshima H and Hunter T (1994) P27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to P21. *Cell* **78:**67–74.

Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, et al. (2002) Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor P27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nat Med* **8:**1136–1144.

Wosikowski K, Schuurhuis D, Johnson K, Paull KD, Myers TG, Weinstein JN, and Bates SE (1997) Identification of epidermal growth factor receptor and C-ErbB2 pathway inhibitors by correlation with gene expression patterns. *J Natl Cancer Inst* **89:**1505–1515.

Yarden Y and Sliwkowski MX (2001) Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2:**127–137.

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