Inhibition of Insulin-like Growth Factor–Binding Protein-3 Signaling through Sphingosine Kinase-1 Sensitizes Triple-Negative Breast Cancer Cells to EGF Receptor Blockade

Janet L. Martin, Hasanthi C. de Silva, Mike Z. Lin, Carolyn D. Scott, and Robert C. Baxter

Abstract

The type I EGF receptor (EGFR or ErbB1) and insulin-like growth factor–binding protein-3 (IGFBP-3) are highly expressed in triple-negative breast cancer (TNBC), a particularly aggressive disease that cannot be treated with conventional therapies targeting the estrogen or progesterone receptors (ER and PR), or HER2. We have shown previously in normal breast epithelial cells that IGFBP-3 potentiates growth-stimulatory signaling transduced by EGFR, and this is mediated by the sphingosine kinase-1 (SphK1)/sphingosine 1-phosphate (S1P) system. In this study, we investigated whether cotargeting the EGFR and SphK1/S1P pathways in TNBC cells results in greater growth inhibition compared with blocking either alone, and might therefore have novel therapeutic potential in TNBC. In four TNBC cell lines, exogenous IGFBP-3 enhanced ligand-stimulated EGFR activation, associated with increased SphK1 localization to the plasma membrane. The effect of exogenous IGFBP-3 on EGFR activation was blocked by pharmacologic inhibition or siRNA-mediated silencing of SphK1, and silencing of endogenous IGFBP-3 also suppressed EGF-stimulated EGFR activation. Real-time analysis of cell proliferation revealed a combined effect of EGFR inhibition by gefitinib and SphK1 inhibition using SKi-II. Growth of MDA-MB-468 xenograft tumors in mice was significantly inhibited by SKi-II and gefitinib when used in combination, but not as single agents. We conclude that IGFBP-3 promotes growth of TNBC cells by increasing EGFR signaling, that this is mediated by SphK1, and that combined inhibition of EGFR and SphK1 has potential as an anticancer therapy in TNBC in which EGFR and IGFBP-3 expression is high.

Introduction

Approximately, 15% of breast tumors are classified as triple-negative breast cancers (TNBC), a term that denotes their lack of estrogen receptor (ER) and progesterone receptor (PR), and nonamplification of the HER2. These tumors, which are particularly aggressive and tend to occur with higher frequency in young women, cannot be targeted by therapies that depend on the expression of functional ER, PR, and HER2. Expression of type I EGF receptor (EGFR), a receptor tyrosine kinase that transduces potent proliferative and cell survival signals in many malignancies including breast cancer (1, 2), is typically upregulated in TNBC (3), but clinical trials have not shown significant benefit from single-line targeting of EGFR in TNBC (4).

Insulin-like growth factor–binding protein-3 (IGFBP-3) is one of six proteins that bind the growth factors IGF-I and -II with high affinity, and modulate their potent proliferative and cell-survival effects mediated by the type I IGF receptor (IGF-IR). IGFBP-3 also exerts growth-inhibitory activity independent of modulating IGF-IR activation by IGFs, and in some tissues this results in suppression of tumor growth and metastasis (5, 6). In contrast, studies from a number of groups have demonstrated growth-promoting activity of IGFBP-3 in vitro (7–11) and elevated gene expression of IGFBP3 occurs in a range of malignancies (12–14). In human breast tumors, expression of IGFBP-3 is correlated with markers of poor prognosis such as ER and PR negativity, S-phase fraction, and tumor size (15–18). These clinical observations were recapitulated in a xenograft tumor model in which T47D cells expressing IGFBP-3 as a result of cDNA transfection developed larger tumors than control cells in nude mice (19).

Several mechanisms underlying the stimulation of breast cancer cell growth by IGFBP-3 have been described by our laboratory, including the prevention of inhibitory nuclear receptor signaling (20) and the promotion of cell survival by autophagy (21). Our studies have also revealed that in MCF-10A mammary epithelial cells...
IGFBP-3 potentiates ligand-stimulated activation of EGFR (11, 22). The effects of IGFBP-3 on EGFR activation required sphingosine kinase-1 (SphK1), which catalyzes the conversion of sphingosine to sphingosine 1-phosphate (S1P), and were mimicked by exogenous S1P, suggesting that S1P mediates potentiation of the EGFR signaling pathway by IGFBP-3 in breast epithelial cells. These findings imply that functional blockade of SphK pathway signaling has the potential to block IGFBP-3 stimulatory bioactivity.

Breast cancer cell lines that exhibit molecular features of TNBC, such as the Hs578T and MDA-MB-231 cell lines, reflect the clinical disease with high expression of EGFR and IGFBP-3 (23, 24). In view of this, we have investigated the role of IGFBP-3 and SphK1 in EGFR signaling in TNBC cells, and the potential efficacy of cotargeting the IGFBP-3/SphK and EGFR pathways as a novel therapy for the treatment of these cancers.

Materials and Methods

Materials

Tissue culture reagents and plasticware were from Trace Biosciences and Nunc. Bovine insulin, EGF, hydrocortisone, and α-tubulin antibody were purchased from Sigma. Antibodies against phospho-Tyr1068 EGFR and total EGFR, phospho-Ser473 AKT and total AKT, and phospho-Thr202/Tyr204 extracellular signal-regulated kinase (ERK)1/2 and total ERK1/2 were purchased from Cell Signaling Technology and their specificity verified previously (22). SphK1 antibody (ab16491) was from Abcam, ER-α antibody was from Epitomics, and aquaporin 1 (AQP1) was from Santa Cruz Biotechnology. Recombinant human IGFBP-3 was expressed in human 911 retinoblastoma cells using an adenoviral expression system and purified as previously described (25). SphK inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole (SKi-II; ref. 26) was from Calbiochem. Gefitinib was purchased from Enzo Life Sciences, and inhibitor 2-((N-(6-(1H-indol-2-yl)-4H-3,1-benzoxazin-2-yl)amino)phenyl)sulfonyl)acetamide (SB746322) was from Selleck Chemicals.

Cell culture

The following breast cancer cell lines were purchased from the American Type Culture Collection (ATCC): BT549, MDA-MB-231, MDA-MB-436, MDA-MB-468, Hs578T, MCF-7, T47D, and ZR-75-1. Identity of Hs578T, T47D, and ZR-75-1 cells, which were obtained from ATCC between 2001 and 2005, was confirmed by short-tandem repeat profiling by CellBank Australia in December 2012. Cryopreserved stocks of other cell lines (purchased in 2010 from ATCC) were established within 1 month of receipt, and fresh cultures for use in experiments were established from these stocks every 2 to 3 months. All lines were maintained in RPMI-1640 medium containing 5% FBS and 10 μg/mL bovine insulin in a humidified 5% CO2 atmosphere at 37°C, and were negative for Mycoplasma contamination.

qRT-PCR

IGFBP3 and expression was monitored by qRT-PCR using TaqMan Gene Expression Assays (Applied Biosystems). Total RNA was isolated from breast cancer cells using TRIzol reagent (Life Technologies) and reverse-transcribed using SuperScript III First Strand Synthesis SuperMix (Invitrogen) according to the manufacturer’s protocols. TaqMan assays for IGFBP-3 (Hs00181211_m1) and SphK1 (Hs00184211_m1) were performed using a Rotor-Gene 3000 thermal cycler (Corbett Research), with hydroxyethylbilane synthase (HMBS; Hs00609297_m1) amplification used as internal control. Results were analyzed using the Rotor-Gene 6 software.

EGFR activation assays

Cells were plated into 12-well plates at 2.5 × 105 cells per well, and maintained in growth medium for 48 hours, then medium without insulin for 24 hours. Fresh medium containing IGFBP-3 with or without inhibitors was added for 16 hours, then EGF was added directly to cells to give final concentrations as indicated for individual experiments. Incubations were continued at 37°C for 10 minutes, then cells were washed with ice-cold PBS and lysed directly into Laemmli sample buffer [62.5 mmol/L Tris–HCl pH 6.8, containing 20 g/L SDS, 100 mL/L glycerol, 1 g/L bromphenol blue, and 50 mmol/L dithiothreitol (DTT)] at 4°C for 10 minutes. Lysates were transferred to ice-cold Eppendorf tubes, and stored at −80°C until Western blot analysis.

Plasma membrane isolation

Fractions containing plasma membranes were isolated to monitor redistribution of SphK1 in response to treatment with IGFBP-3. Briefly, treated cells were washed two to three times with cold PBS, and then harvested in homogenization buffer [20 mmol/L HEPES pH 7.6 containing 250 mmol/L sucrose, 2 mmol/L DTT, 2 mmol/L EDTA, 2 mmol/L EGTA, and protease inhibitor cocktail (Roche)]. Cells were homogenized in a Teflon-glass homogenizer using 50 to 60 strokes and then incubated on ice for 20 minutes. Cellular debris and other organelles...
and nuclei were pelleted by centrifugation at 10,000 x g at 4°C for 20 minutes, and the supernatant containing cytosolic and membrane fractions was then centrifuged at 100,000 x g for 1 hour. The final membrane pellet was resuspended in radioimmunoprecipitation assay buffer (RIPA) lysis buffer (50 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA) containing 0.1% Triton X-100 and stored at −80°C until Western blot analysis.

**Western blotting**

Cell lysates were prepared for SDS-PAGE analysis by sonication for 15 seconds on ice, heating at 95°C for 8 minutes, and centrifugation for 1 minute at 12,000 rpm. Samples were fractionated by 7.5% SDS-PAGE and then proteins were transferred to Hybond C nitrocellulose (Amersham) at 115 mA for 2 hours. Filters were blocked in 50 g/L skim milk powder in TBS-T (TBS with Tween-20: 10 mmol/L Tris, 150 mmol/L NaCl, pH 7.4 containing 1 mL/Tween-20) and probed with primary antibodies diluted in TBS-T containing 10 g/L BSA at 4°C for 16 hours. Filters were washed in cold TBS-T, and incubated with the appropriate horseradish peroxidase–labeled secondary antibody for 1 to 2 hours at room temperature. Washed filters were developed by ECL using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). Total and phosphorylated proteins were analyzed on replicate blots, and filters were reprobed with α-tubulin antibody as a loading control. Bands were visualized using a FUJIFILM Luminescent Image Analyzer LAS-300, and quantified using Image Guage software (Science Lab 2004).

**Cell proliferation assays**

Real-time assessment of cell proliferation over 4 to 6 days was carried out using the IncuCyte Imaging System (Essen Biosciences). Cells (1 x 10^3/well for MDA-MB-231, MDA-MB-436, and Hs578T, and 5 x 10^3 cells/well for MDA-MB-468) were dispensed into 96-well plates in complete medium and then incubated overnight before changing to fresh medium containing 5% FBS and inhibitors. Plates were transferred to the IncuCyte apparatus, and incubations were continued over 72 to 136 hours, depending on the cell line. Images (4/well) were collected every 2 hours over this time. Cell proliferation over 5 days was also measured using the CyQUANT NF Cell Proliferation Assay (Molecular Probes, Life Technologies). Resuspended cells (5 x 10^5) were dispensed into 96-well plates in 200 µL complete medium and allowed to adhere for 24 hours at 37°C. Media were changed to 100 µL RPMI containing 5% FBS and inhibitors, and incubations were continued for 5 days before quantitication.

**Tumor growth in vivo**

Animal studies were approved by the Institutional Animal Care and Ethics Committee (ACEC protocol 1105-010A). Tumors were established in 8-week-old female athymic BALB/c-Foxn1nu/Arc mice (Animal Resources Centre) by injecting MDA-MB-468 cells (5 x 10^6 in 100 µL mixed with 50 µL Matrigel) subcutaneously between the scapulae. Tumor growth was monitored by caliper measurement weekly until tumors reached a volume of approximately 150 mm^3 (calculated as L x W^2/2), when drug treatment was initiated. Groups of 10 animals were injected intraperitoneally three times weekly with vehicle [dimethyl sulfoxide (DMSO)], gefitinib (75 mg/kg), SKI-II (50 mg/kg), or combined SKI-II and gefitinib at these doses, in a volume of 50 µL. Treatment and tumor measurements were continued until the volume of tumors in control (vehicle) mice was 400 to 500 mm^3. Animals were euthanized, and tumors removed, weighed, and snap-frozen in liquid nitrogen. Tumors were processed for Western blot analysis by homogenization in lysis buffer (10 mmol/L Tris, pH 8.0 containing 137 mmol/L NaCl, 10 g/L Triton X-100, 10% glycerol, and protease, and phosphatase inhibitors) using 10 strokes of a Teflon-glass homogenizer. Samples were sonicated and centrifuged, and the supernatant transferred to fresh tubes for storage at −80°C. Protein was quantified and 200 µg loaded onto replicate gels for immunoblot analysis of total and phosphorylated EGFR, AKT, and ERK1/2 as described above.

**Statistical analysis**

All in vitro experiments were performed a minimum of three times and are shown as quantified data (mean ± SEM) pooled from the three experiments, unless indicated otherwise. Statistical analysis (ANOVA with Bonferroni post hoc test) was performed using Prism 4 for Macintosh (GraphPad Software, Inc.).

**Results**

**Molecular features of TNBC and ER− cell lines**

We have previously shown high levels of IGFBP-3 expression in ER− tumors and an ER− breast cancer cell line, Hs578T, and low levels in ER+ tumors and cells (24). To extend this, we screened eight human breast cancer cell lines, five ER− and representative of TNBC, and three ER+, for IGFBP-3 gene and protein expression. The cell lines were selected for analysis based on their documented lack of expression of both ER and PR, and no amplification of HER2 (23). Both IGFBP-3 gene expression (measured by qRT-PCR; Fig. 1A, top) and secreted protein levels (measured by RIA; Fig. 1A, bottom) were markedly higher in ER− cell lines than ER+ cell lines, which had levels of IGFBP-3 protein below the limit of detection of the assay. The highest expressing line, MDA-MB-468, had 1,000-fold higher levels of IGFBP-3 mRNA than the lowest ER+ cell line, ZR-75-1.

The expression of proteins involved in EGFR and IGFBP-3 signaling was characterized in these cell lines maintained in 5% FBS (Fig. 1B). Expression of EGFR was readily apparent, although variable, in the five TNBC cell lines, but was virtually undetectable in the ER+ cell lines MCF-7, T47D, and ZR-75-1. Like IGFBP-3 expression, EGFR expression was highest in the MDA-MB-468 cell line, and phosphorylation of EGFR at tyrosine 1068...
was apparent in only these cells under serum-containing (Fig. 1B) and serum-free (data not shown) conditions. Expression and activation (phosphorylation) of AKT and ERK1/2, two key signaling intermediates downstream of EGFR, were apparent in all cell lines under serum-replete (Fig. 1B) and serum-free (data not shown) conditions.

Our previous study demonstrated that IGFBP-3 potentiation of EGFR activation requires SphK1 (22). Analysis of SphK1 by immunoblot of cell lysates revealed its...
expression by all cell lines, with multiple bands ranging in mass from approximately 40 to 70 kDa variably expressed in the different cell lines (Fig. 1B). In view of literature suggesting the existence of only three SphK1 forms (27), the presence of additional bands suggested that the antibody used to detect SphK1 (Abcam #16419) was also reacting nonspecifically with unrelated proteins in the cell lysates. To determine the bands corresponding specifically to SphK1 in TNBC cell lines, and thereby the expression profile of this protein in TNBC, we applied siRNA-mediated knockdown of SphK1 in the TNBC cell lines, reasoning that bands which were reduced in intensity represented proteins immunologically related to SphK1. qRT-PCR confirmed that two siRNA constructs targeting SphK1 reduced SphK1 gene expression approximately 90% in MDA-MB-231, MDA-MB-436, and MDA-MB-468 cells, and by approximately 75% in Hs578T (Fig. 1C, top). Western blot analysis of SphK1 in lysates from the cells (Fig. 1C, bottom) revealed that a number of bands were reduced or lost in the TNBC cell lines in siRNA-transfected cells, including bands of approximately 42 kDa (white arrows in Fig. 1C), 51 kDa (black arrows), and 44 kDa (gray arrows), which likely represent SphK1a, and 86- and 14-amino acid N-terminally extended forms of SphK1 (SphK1b and SphK1c), respectively (27). These were differentially expressed in the different cell lines, with SphK1a expressed by all cell lines, SphK1b expressed by MDA-MB-436, MDA-MB-468, and Hs578T, and SphK1c expressed in the MDA-MB-231, MDA-MB-436, and Hs578T cell lines. An uncharacterized 65-kDa protein was also reduced by SphK1 silencing in MDA-MB-436, MDA-MB-468, and Hs578T. Two proteins of approximately 45 and 53 kDa, indicated by asterisks in Fig. 1C, were not decreased by SphK1 knockdown. There was no correlation between either the form of SphK1 expressed or its total levels, and ER status of the cell lines (Fig. 1B).

**Exogenous IGFBP-3 potentiates EGFR activation in TNBC lines and induces SphK1 translocation to the plasma membrane**

To determine whether IGFBP-3 enhances the effects of EGF in TNBC cell lines, EGFR phosphorylation at Y1068 was assessed in four TNBC cell lines treated overnight with IGFBP-3 before stimulation with EGF for 10 minutes. As shown in Fig. 2A, preincubation with IGFBP-3 enhanced EGFR-stimulated EGFR phosphorylation in MDA-MB-231, MDA-MB-436, and Hs578T cell lines, with the greatest enhancement (2- to 2.5-fold) in the MDA-MB-231 and Hs578T lines. Although a trend toward slightly increased EGFR phosphorylation in response to IGFBP-3 was apparent in the MDA-MB-468 cells, this was not statistically significant. In the absence of EGF, IGFBP-3 did not stimulate EGFR phosphorylation or expression in any cell line (Supplementary Fig. S1).

Phosphorylation-induced translocation of SphK1 to the plasma membrane is essential for generation of S1P (28). To assess activation of SphK1 in response to IGFBP-3, we analyzed SphK1 in membranes isolated from cells treated with IGFBP-3, using the transmembrane water channel protein AQP1 as a loading control (Fig. 2B). Western blot analysis of membrane fractions revealed a transient increase in SphK1a (42 kDa), which is expressed by all the TNBC cell lines, peaking 30 to 60 minutes after addition of IGFBP-3, and declining thereafter in MDA-MB-231 and MDA-MB-436 cells. A slightly delayed time-course and blunted response was apparent for Hs578T cells, with a significant increase apparent only after 2 hours. The very low level of SphK1 expression in MDA-MB-468 cells (as shown in Fig. 1) precluded this analysis in that cell line. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a cytoplasmic protein, was not detected in these fractions, indicating no cytoplasmic contamination of the membrane fractions (data not shown). There was no clear evidence of translocation of either SphK1b (51 kDa) or SphK1c (44 kDa) in response to IGFBP-3 (Supplementary Fig. S2), although both are expressed in MDA-MB-436 and Hs578T. The increase in membrane SphK1 over this time-course was dependent on IGFBP-3, as shown for MDA-MB-231 cells in Supplementary Fig. S2, with similar results obtained for MDA-MB-436 cells (data not shown). These data suggest that IGFBP-3 is activating SphK1 in these cells at least in part by inducing its relocation to the plasma membrane.

Silencing of IGFBP-3 in mammary epithelial cells reduces the ability of EGF to stimulate EGFR phosphorylation, indicating an influence of endogenous IGFBP-3 on receptor activation in these cells (22). To investigate whether endogenous IGFBP-3 is also required for optimal EGFR activation in breast cancer cells that highly express the protein, IGFBP-3 was silenced using either of two siRNAs, and EGFR phosphorylation was determined after stimulation with EGF. RIA and Western blot analysis of conditioned medium and cell lysates confirmed >90% knockdown of IGFBP-3 protein expression in each cell line (data not shown). As shown in Fig. 2C, the ability of EGF to stimulate EGFR phosphorylation was significantly reduced in all cell lines when IGFBP-3 was silenced compared with nonsilencing control. We also found that basal EGFR phosphorylation in MDA-MB-468 was reduced in IGFBP-3 knockdown cells in the absence of exogenous EGF (Fig. 2C). Thus, the sensitivity of EGFR to stimulation by EGF in these TNBC cell lines is enhanced by endogenous IGFBP-3.

**SphK1 inactivation or silencing blocks IGFBP-3 potentiation of EGFR activation in TNBC cell lines**

To demonstrate that SphK1 is involved in the effect of IGFBP-3 on EGFR activation in TNBC cells, downregulation of its expression and activity was achieved by siRNA-mediated silencing and pharmacologic inhibition, respectively. As shown in Fig. 3A, transfection with an siRNA construct targeting SphK1, which reduced its mRNA and protein levels by 75% to 90% (shown in Fig. 1C) was accompanied by a loss in the potentiation of EGF-stimulated EGFR phosphorylation elicited by preincubation...
with IGFBP-3. Similar results were seen for a second SphK1 siRNA (data not shown). Consistent with this, SKi-II, an inhibitor of SphK activity (26), blocked the enhancement of EGF-stimulated EGFR phosphorylation induced by IGFBP-3 in MDA-MB-231, MDA-MB-436, and Hs578T cells (Fig. 3B). Taken together, these data indicate
that SphK1 expression and activity are required for IGFBP-3 to enhance EGFR signaling in TNBC cells.

**SKi-I induces degradation of SphK1a in TNBC cell lines**

Gefitinib has been reported to inhibit expression of SphK1 in glioblastoma cells (29), and SKi-II has been shown in other cell types to induce degradation of SphK1 by targeting it to the ubiquitin-proteasomal degradation pathway (27). In TNBC cells, SKi-II (1–10 μmol/L) alone significantly reduced SphK1a in all cell lines (P < 0.001) with the greatest decrease in MDA-MB-436 cells (~80%) and smallest in Hs578T (~50%; Fig. 4A). Gefitinib alone had a significant inhibitory effect on SphK1a expression in MDA-MB-436 cells (to 77% ± 6.3% of control levels; P < 0.001), but there was no combined effect of gefitinib and SKi-II in this or any other cell line (Fig. 4A). To demonstrate that the loss of SphK1a induced by SKi-II was due to its proteolysis, treatment with SKi-II was carried out in the presence of the proteasome inhibitor MG132. As shown in Fig. 4B, MG132 increased SphK1a in the absence of SKi-II, and prevented its SKi-II–induced loss in all cell lines. Similarly, SKi-II decreased, and MG132 increased, SphK1b and SphK1c in those cells expressing these forms of the protein, although the changes were not as marked as for SphK1a (data not shown). The slight decrease in SphK1a elicited by gefitinib in MDA-MB-436 (shown in Fig. 4A) was also reversed by MG132 (data not shown).
This implies that SphK1 turnover involves proteasomal degradation, and that this is increased by SKi-II.

Effect of combined EGFR and SphK1 inhibition of proliferation of TNBC cells

The SphK and EGFR signaling systems act both independently and cooperatively to stimulate cell proliferation and survival in many normal and malignant cell types (30), raising the possibility that targeting these two systems together in TNBC cells, which express IGFBP-3 highly, will have greater effect than blocking either alone. Therefore, the functional consequences of single and combined blockade of the EGFR and SphK1 signaling pathways was determined in the four TNBC cell lines. Initially, the sensitivity to gefitinib and SKi-II in medium containing 5% FBS was determined using CyQUANT, a cell proliferation assay that measures cellular DNA content. As shown in Supplementary Fig. S3A, SKi-II alone inhibited proliferation of all cell lines over a range of doses, with MDA-MB-468 exhibiting greatest sensitivity, and Hs578T least sensitivity, to its effects. Gefitinib alone was strongly inhibitory to MDA-MB-436 and MDA-MB-468 cells, and had an additive effect with low concentrations of SKi-II in all cell lines. The pattern of inhibition in response to gefitinib alone was similar, and a clear additional inhibitory effect was apparent when SKi-II was included at low gefitinib concentrations (Supplementary Fig. S3B).

To study the effects of the EGFR kinase and SphK1 inhibitors in more detail, real-time proliferation experiments were conducted using low gefitinib and SKi-II doses, selected according to the relative sensitivity of the individual cell lines to these agents determined by using the CyQUANT assay. Cells were sparsely plated in 96-well plates, changed to medium containing inhibitors 16 hours later, and proliferation over the following 72 to 140 hours was imaged using an IncuCyte apparatus. As shown in Fig. 5A, at the low doses chosen gefitinib had no significant inhibitory effect when used alone in any TNBC cell line. SKi-II significantly inhibited growth of MDA-MB-231 (from 76 hours onwards) and MDA-MB-436 (at the latest time point, 128 hours). Remarkably, in view of the modest effects when the inhibitors were used separately, the combination of gefitinib and SKi-II virtually abolished cell proliferation in all cell lines, with a significant effect compared with...
control apparent from 28 hours for Hs578T, 52 hours for MDA-MB-468, 56 hours for MDA-MB-231, and 68 hours for MDA-MB-436 (\(P < 0.01\)).

In view of the profound combined effect of gefitinib and SKi-II on the proliferation of TNBC cells, we investigated the effects of these agents on EGF-stimulated activation of EGFR, and two key survival and proliferative signaling pathways, AKT and ERK1/2. As shown in Fig. 5B, EGFR phosphorylation at Y1068 was markedly increased by exogenous EGF in MDA-MB-231, MDA-MB-436, and Hs578T cell lines, with no apparent increase in EGFR phosphorylation above the high basal level seen under unstimulated conditions in the MDA-MB-468 line (Fig. 5B). Gefitinib alone inhibited EGFR phosphorylation in all cell lines, but the inclusion of SKi-II further inhibited EGFR phosphorylation only in MDA-MB-468 at the highest dose (3 \(\mu\)mol/L) of gefitinib (\(P < 0.05\)). The four cell lines showed differing patterns of activation of ERK1/2 and AKT in response to EGF (Fig. 5B).
effect on ERK1/2, the opposite pattern of activation was apparent in MDA-MB-436 and MDA-MB-468, and the two proteins showed a similar degree of activation of Hs578T cells. Gefitinib alone had the greatest inhibitory effect on those pathways most sensitive to EGF stimulation: AKT in MDA-MB-231, pERK1/2 in MDA-MB-436 and MDA-MB-468, and similar inhibition of these pathways in Hs578T (Fig. 5B). SKi-II did not enhance the inhibitory effect of gefitinib on AKT or ERK1/2 phosphorylation in any cell line. The quantified data from three to six similar experiments are given in Supplementary Fig. S4. Collectively these data indicate that the remarkable combined inhibitory effect of SKi-II and gefitinib on growth of these TNBC cell lines shown in Fig. 5A, cannot be explained by effects on these signaling intermediates.

The combination of gefitinib and SKi-II inhibits growth of MDA-MB-468 xenograft tumors

The profound combined effect of EGFR and SphK1 on inhibition of TNBC cells in vitro provided proof-of-principle that cotargeting these pathways may have therapeutic potential for the treatment of aggressive TNBC breast tumors. To investigate this in vivo, MDA-MB-468 cells were established as xenograft tumors in immunocompromised mice, then treated with vehicle (50 μL DMSO), gefitinib alone (75 mg/kg), SKI-II alone (50 mg/kg), or a combination of the two at these doses given as a single injection intraperitoneally three times weekly. Tumor volumes were measured immediately before treatment was initiated and then at 5 and 11 days of treatment. Experiments were terminated after 11 days due to the largest tumors in control mice reaching the maximum size stipulated in the animal ethics protocol (500 mm³). Before starting treatment, mice were randomized into treatment groups of 9 to 10 mice per group. The mean ± SEM of tumor volumes in the groups before treatment were: control, 146.1 ± 20.2 mm³; gefitinib, 138.5 ± 18.6 mm³; SKI-II, 151.6 ± 20.7 mm³; and combination, 142.1 ± 21.5 mm³ (P = ns). As shown in Fig. 6A, which depicts the mean ± SEM change in tumor volume of mice after 5 and 11 days of treatment, the combination of SKI-II and gefitinib markedly inhibited tumor growth within 5 days of treatment, and this inhibitory effect was maintained until the termination of the experiment (P = 0.011, by repeated measures). Under these conditions, neither SKI-II (P = 0.477) nor gefitinib (P = 0.82) alone significantly inhibited tumor growth compared with control. There was no significant effect of any treatment on body weight at any time point (data not shown). At the conclusion of the experiment, mean ± SEM of tumor volumes of the combination group was 243.3 ± 22.84 mm³, compared with control of 398.1 ± 48.24 mm³ (P < 0.05). The tumor volumes of the gefitinib (331.8 ± 49.7 mm³) and SKI-II (309.7 ± 35.4 mm³) groups did not differ significantly from control.

Western blot analysis of activation of EGFR, AKT, and mitogen-activated protein kinase (MAPK) in excised tumors (Fig. 6B) revealed that, similar to the effects of gefitinib and SKI-II on MDA-MB-468 cells in vitro, EGFR and ERK1/2 phosphorylation in tumors was markedly inhibited by gefitinib alone, with little effect on AKT phosphorylation. SKI-II alone did not significantly affect phosphorylation of any of these proteins. Although the addition of SKI-II to the gefitinib treatment reduced EGFR phosphorylation further as compared with gefitinib alone, this did not reach statistical significance (P = 0.06).
Discussion

This study sought to investigate the potential efficacy of cotargeting growth-stimulatory signaling pathways of two proteins that are highly expressed in TNBC: EGFR and IGFBP-3. Because many such cancers express EGFR highly (3, 31, 32), there has been a major interest in drugs that target this pathway, for use either as a single agent or in combination with conventional chemotherapy. In general, EGFR inhibition as a monotherapy for TNBC has been disappointing (33, 34) and a number of combined targeted treatments are currently under investigation.

The decision to combine EGFR blockade with inhibition of an IGFBP-3 signaling pathway drew on observations made by us and others of high IGFBP-3 and EGFR expression in ER0 breast tumors and cancer cells compared with ER0 (3, 15, 16, 24, 35, 36) and our previous studies showing potentiation of EGFR signaling by IGFBP-3 in untransformed breast epithelial cells (11, 22). Although targeting IGFBP-3 itself would arguably be a more direct means of blocking its growth-stimulatory signaling, its function as a key regulator of the IGF axis makes this approach technically implausible in vivo. Having identified the SphK1/S1P system as the mediator of IGFBP-3’s stimulatory effects on EGFR signaling in normal breast cells (22), we regarded this system as a logical target for inhibiting IGFBP-3 stimulatory bioactivity in breast cancer cells.

Three of the four TNBC cell lines studied responded to exogenous IGFBP-3 with significantly increased EGF-stimulated EGFR phosphorylation and, importantly, endogenous IGFBP-3 was also shown to modulate EGFR activation in TNBC cells, as demonstrated by a reduction in EGF-stimulated EGFR phosphorylation when IGFBP-3 was silenced. This observation alone places IGFBP-3 clearly in the pathway of EGFR action in TNBC cells. The effects of IGFBP-3 on EGFR phosphorylation in TNBC cell lines required SphK activity as SKi-II, a dual SphK1 and SphK2 inhibitor, blocked enhancement of EGFR phosphorylation by IGFBP-3. SphK1 mediates these effects of IGFBP-3 in TNBC cells, because when SphK1 expression was silenced using siRNA, IGFBP-3 no longer potentiated EGF-stimulated EGFR phosphorylation. A role for SphK2 in the effects of IGFBP-3 on EGFR signaling seems unlikely, as siRNA-mediated silencing of SphK2 did not prevent IGFBP-3-potentiating EGFR activation in phenotypically normal breast epithelial cells (22).

Exogenous IGFBP-3 upregulates both SphK1 expression and activity in breast epithelial cells (22), but here we found no clear correlation between IGFBP-3 expression and either the total amount of SphK1 or the isoforms expressed in breast cancer cells. This suggests that endogenous IGFBP-3 is not a dominant regulator of SphK1 expression in breast cancer cells and that its effects on EGFR signaling relate to its modulation of SphK1 activity rather than expression. Supporting this, exogenous IGFBP-3 increased SphK1 in the membrane fractions of TNBC cells, demonstrating that in these cells IGFBP-3 increases the activity of SphK1. Translocation of SphK1 to the plasma membrane is a process clearly linked with the generation of S1P (37). Although formation of S1P in response to IGFBP-3 was not explicitly shown in the present study, we and others have previously shown induction of SphK activity and increased S1P levels in response to IGFBP-3 in breast and other cell types (22, 38, 39). The mechanism by which it does so remains unknown.

Analysis of SphK1 expression by the TNBC cell lines revealed a number of bands by Western blot analysis, ranging in size from approximately 42 to 65 kDa. Silencing of SphK1 revealed that some of these bands represented proteins not immunologically related to SphK1, presumably reacting nonspecifically with the antibody. However, proteins of mass consistent with SphK1a, SphK1b, and SphK1c were expressed by TNBC, though not all species were detected in all cell lines. The biologic significance of different SphK1 forms is not known, although it has been suggested that they exhibit different subcellular localization (40) and may therefore have functional specificity. The SphK1 variants were all decreased by SKi-II and, as in other cell types (27, 41, 42), this was reversed by the proteasome inhibitor MG132, implying their degradation via ubiquitin-proteasomal pathways. MG132 also increased various SphK1 forms to levels above those in untreated cells, suggesting proteasome regulation of the enzyme under basal conditions. The functional consequence of SphK1 degradation in TNBC cells is not clear, but SKi-II–induced degradation of SphK1 has been reported to increase apoptosis of androgen-sensitive prostate cancer cells (27), perhaps by shifting the sphingolipid rheostat toward the accumulation of proapoptotic precursors of S1P, such as sphingosine and ceramide (43). Notably, IGFBP-3 has been shown to enhance the apoptotic effects of C2 ceramide in breast cancer cells (44), suggesting that there may be increased apoptotic activity in breast cancer cells in which there is accumulation of ceramide if those cells also express IGFBP-3. We showed previously that functional blockade of EGFR restored sensitivity to growth-inhibitory effects of ectopically expressed IGFBP-3 in ER0 breast cancer cells (19), which would be another reason to target the SphK1 pathway rather than IGFBP-3 itself in TNBC in which IGFBP-3 is highly expressed.

Although the sensitivity of the four TNBC cell lines to gefitinib and SKi-II was variable, the combination of gefitinib and SKi-II almost completely blocked proliferation of all lines when the two inhibitors were used at concentrations that alone had little or no inhibitory effect. Importantly, when the combination of SKi-II and gefitinib was tested in an MDA-MB-468 xenograft tumor model, it significantly inhibited tumor growth under conditions in which neither gefitinib nor SKi-II alone had a significant effect. The mechanisms underlying these combined effects are still to be identified, with no apparent involvement of the key growth-regulatory ERK1/2 or AKT pathways either in vitro or in vivo.

Compared with EGFR, interest in SphK1 as a molecular target in cancer is relatively recent but an increasing
number of pharmacologic SphK inhibitors are being evaluated in preclinical settings. The growth of MDA-MB-468 xenograft tumors was shown to be inhibited by ABC294640, an SphK2-specific inhibitor, when used as a single-line agent (45). Acquired resistance to chemotherapeutic drugs, hormonal therapies, and growth factor receptor inhibitors, including EGFR inhibitors, has been linked with overexpression of SphK1 in a number of malignancies (46–50), providing a clear rationale for the use of SphK inhibitors in the clinic as adjuvant therapies. Our study has shown potent antiproliferative effects of dual inhibition of EGFR and SphK1 in cell lines representing a subset of breast cancers for which there is currently a paucity of treatments, and has demonstrated in a preclinical study proof-of-principle that this combination has therapeutic potential. Further optimization of dosing schedules may improve the observed effects. We propose that a regimen of combining SphK1 inhibition with EGFR inhibition constitutes a novel therapeutic option in TNBC that could be rapidly implemented in the clinic.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J.L. Martin, R.C. Baxter
Development of methodology: M.Z. Lin, C.D. Scott
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. Martin, H.C. de Silva, M.Z. Lin, C.D. Scott, R.C. Baxter
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. Martin, H.C. de Silva, M.Z. Lin, R.C. Baxter
Writing, review, and/or revision of the manuscript: J.L. Martin, M.Z. Lin, C.D. Scott, R.C. Baxter
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.C. de Silva, M.Z. Lin
Study supervision: J.L. Martin, R.C. Baxter

Grant Support
R.C. Baxter and J.L. Martin received a grant from the Cancer Council NSW (RG 11–09).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 14, 2013; revised November 15, 2013; accepted December 1, 2013; published OnlineFirst December 13, 2013.

References
www.aacrjournals.org Mol Cancer Ther; 13(2) February 2014


Molecular Cancer Therapeutics

Inhibition of Insulin-like Growth Factor–Binding Protein-3 Signaling through Sphingosine Kinase-1 Sensitizes Triple-Negative Breast Cancer Cells to EGF Receptor Blockade

Janet L. Martin, Hasanthi C. de Silva, Mike Z. Lin, et al.

Mol Cancer Ther  Published OnlineFirst December 12, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0367

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2013/12/12/1535-7163.MCT-13-0367.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.