Inhibition of insulin-like growth factor binding protein-3 signaling through sphingosine kinase 1 sensitizes triple-negative breast cancer cells to EGF receptor blockade.

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Abbreviations: IGFBP-3, insulin-like growth factor binding protein-3; EGFR, epidermal growth factor receptor; ER, estrogen receptor; SphK, sphingosine kinase; S1P, sphingosine 1-phosphate; TNBC, triple-negative breast cancer.

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ABSTRACT

The type 1 epidermal growth factor 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 co-targeting the EGFR and SphK1/S1P pathways in TNBC cells causes increased growth inhibition compared to 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 pharmacological 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 where 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 ER and PR, and non-amplification of the human EGF receptor 2 (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 1 epidermal growth factor 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 1 IGF receptor (IGF1R). IGFBP-3 also exerts growth-inhibitory activity independent of modulating IGF1R activation by IGFs, and in some tissues this results in suppression of tumor growth and metastasis (5,6). By 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 estrogen receptor (ER) and progesterone receptor (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 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 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 sphingosine kinase 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 co-targeting the IGFBP-3/sphingosine kinase 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 (North Ryde, New South Wales, Australia) and Nunc (Roskilde, Denmark). 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 ERK1/2 and total ERK1/2 were purchased from Cell Signaling (Beverley, MA) and their specificity verified previously (22). SphK1 antibody (ab16491) was from Abcam (Walnut, CA), ERα antibody was from Epitomics (Burlingame, CA) and aquaporin 1 (AQP1) was from Santa Cruz. Recombinant human IGFBP-3 was expressed in human 911 retinoblastoma cells using an adenoviral expression system and purified as previously described (25). Sphingosine kinase inhibitor 2-((p-hydroxyanilino)-4-(p-chlorophenyl)thiazole (SKi-II) (26) was from Calbiochem. Gefitinib was purchased from LC Laboratories (Woburn, MA). Electrophoresis and ECL reagents were purchased from Bio-Rad (Hercules, CA), Amrad-Pharmacia (Ryde, New South Wales, Australia) and Pierce (Rockford, IL).

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 (Westmead, NSW, 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 2-3 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.
siRNA-mediated protein knockdown.

Knockdown of protein expression was achieved by electroporation using the following siRNA duplexes from Qiagen (Doncaster, Victoria, Australia): SphK1, Hs_SPHK1_6 and Hs_SPHK1_7; IGFBP-3, Hs_IGFBP3_8 and Hs_IGFBP3_3. To achieve protein silencing, cells were harvested by trypsinization and resuspended at 2.5 x 10^6 cells in 100 μl Amaxa transfection reagent (Lonza Australia Pty Ltd, Mt Waverley, Victoria), then mixed with 1.5 μg silencing siRNA or AllStars negative control siRNA (Qiagen). Nucleoporation was carried out using an Amaxa electroporation unit (Lonza), and cells were transferred to complete medium then plated for specific endpoints as indicated below. Knockdown of protein expression was confirmed by qRT-PCR, in-house IGFBP-3 radioimmunoassay (24) and Western analysis.

Quantitative Real Time-PCR (qRT-PCR).

IGFBP3 and expression was monitored by qRT-PCR using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Total RNA was isolated from breast cancer cells using TRizol reagent (Life Technologies Australia) and reverse-transcribed using Superscript III First Strand Synthesis SuperMix (Invitrogen) according to 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, Mortlake, New South Wales, Australia), with hydroxymethylbilane 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 x 10^5 cells/well, and maintained in growth medium for 48 h, then medium without insulin for 24 h. Fresh medium containing IGFBP-3 with or without inhibitors was added for 16 h, then EGF was added directly to cells to give final concentrations as indicated for individual experiments. Incubations were continued at 37°C for 10 min, then cells were washed with ice-cold PBS and lysed directly into Laemmli sample
buffer (62.5 mM Tris-HCl pH 6.8, containing 20 g/liter SDS, 100 mL/liter glycerol, 1 g/liter bromphenol blue and 50 mM dithiothreitol) at 4°C for 10 minutes. Lysates were transferred to ice-cold Eppendorf tubes, and stored at -80°C until Western 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 2-3 times with cold phosphate buffered saline (PBS), and then harvested in homogenization buffer (20 mM HEPES pH 7.6 containing 250 mM sucrose, 2 mM DTT, 2 mM EDTA, 2 mM EGTA and protease inhibitor cocktail (Roche)). Cells were homogenized in a teflon-glass homogenizer using 50-60 strokes then incubated on ice for 20 min. Cellular debris, and other organelles and nuclei were pelleted by centrifugation at 10,000 x g at 4°C for 20 min, and the supernatant containing cytosolic and membrane fractions was then centrifuged at 100,000 x g for 1 h. The final membrane pellet was resuspended in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA) containing 0.1% Triton X-100 and stored at -80°C until Western analysis.

**Western blotting.**

Cell lysates were prepared for SDS-PAGE analysis by sonication for 15 sec on ice, heating at 95°C for 8 min, and centrifugation for 1 min at 12,000 rpm. Samples were fractionated through 7.5% SDS polyacrylamide gels, then proteins were transferred to Hybond C nitrocellulose (Amersham) at 115 mA for 2 h. Filters were blocked in 50 g/L skim milk powder in TBS-T (tris-buffered saline with Tween-20: 10 mM Tris, 150 mM NaCl, pH 7.4 containing 1 mL/L Tween-20) and probed with primary antibodies diluted in TBS-T containing 10 g/L BSA at 4°C for 16 h. Filters were washed in cold TBS-T, and incubated with the appropriate horseradish peroxidase-labeled secondary antibody for 1-2 h at RT. Washed filters were developed by enhanced chemiluminescence (ECL) using Supersignal West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). Total and phosphorylated
proteins were analyzed on replicate blots, and filters were re-probed with α-tubulin antibody as a loading control. Bands were visualized using a FUJIFILM Luminescent Image Analyzer LAS-300 (Stamford, CT), and quantified using Image Guage software (Science Lab 2004).

**Cell proliferation assays.**

Real-time assessment of cell proliferation over 4-6 days was carried out using the IncuCyte imaging system (Essen Biosciences, Ann Arbor, MI). Cells (1x10^3/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 incubated overnight before changing to fresh medium containing 5% FBS and inhibitors. Plates were transferred to the IncuCyte apparatus, and incubations were continued over 72-136 h, depending on the cell line. Images (4/well) were collected every 2 h over this time. Cell proliferation over 5 days was also measured using the CyQUANT NF cell proliferation assay (Molecular Probes, Life Technologies Australia, Mulgrove, Victoria, Australia). Resuspended cells (5 x 10^3) were dispensed into 96 well plates in 200 μL complete medium and allowed to adhere for 24 h at 37°C. Media were changed to 100 μL RPMI containing 5% FBS and inhibitors, and incubations were continued for 5 days before quantitation.

**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, Perth, Western Australia) 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 ~150 mm^3 (calculated as L x W^2/2), when drug treatment was initiated. Groups of 10 animals were injected i.p. three times weekly with vehicle (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 400-500 mm$^3$. Animals were euthanized, and tumors removed, weighed and snap-frozen in liquid nitrogen. Tumors were processed for Western analysis by homogenization in lysis buffer (10 mM Tris, pH 8.0 containing 137 mM 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±S.E.M.) 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+ve cell lines.

We have previously shown high levels of IGFBP-3 expression in ER-ve tumors and an ER-ve breast cancer cell line, Hs578T, and low levels in ER+ve tumors and cells (24). To extend this we screened 8 human breast cancer cell lines, five ER-ve and representative of TNBC, and three ER+ve, 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: Figure 1A, upper panel) and secreted protein levels (measured by RIA: Figure 1A lower panel) were markedly higher in ER-ve cell lines than ER+ve 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 1000-fold higher levels of IGFBP-3 mRNA than the lowest ER+ve 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 (Figure 1B). Expression of EGFR was readily apparent, although variable, in the five TNBC cell lines, but was virtually undetectable in the ER+ve 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 (Y1068) was apparent in only these cells under serum-containing (Figure 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 (Figure 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 ~40 kDa to ~70 kDa variably expressed in
the different cell lines (Figure 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 non-specifically 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. Quantitative real-time PCR (qRT-PCR) confirmed that two siRNA constructs targeting SphK1 reduced SphK1 gene expression ~90% in MDA-MB-231, MDA-MB-436 and MDA-MB-468 cells, and by ~75% in Hs578T (Figure 1C, upper panel). Western analysis of SphK1 in lysates from the cells (Figure 1C, lower panel) revealed that a number of bands were reduced or lost in the TNBC cell lines in siRNA-transfected cells, including bands of ~42 kDa (white arrows in Figure 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 ~45 kDa and ~53 kDa, indicated by asterisks in Figure 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 (Figure 1B).

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

To determine if 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 min. As shown in Figure 2A, preincubation with IGFBP-3 enhanced EGF-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 towards 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 Figure 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 aquaporin 1 (AQP1) as a loading control (Figure 2B). Western analysis of membrane fractions revealed a transient increase in SphK1a (42 kDa), which is expressed by all the TNBC cell lines, peaking 30-60 min 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 two hours. The very low level of SphK1 expression in MDA-MB-468 cells (as shown in Figure 1) precluded this analysis in that cell line. 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 Figure 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 Figure 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 of conditioned medium and cell lysates confirmed >90% knockdown of IGFBP-3 protein expression in each cell line (data not shown). As shown in Figure 2C, the ability of EGF to stimulate EGFR phosphorylation was significantly reduced in all cell lines when IGFBP-3 was silenced compared with non-silencing control. We also found that basal EGFR phosphorylation in MDA-MB-468 was reduced in IGFBP-3 knockout cells in the absence of exogenous EGF (Figure 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 pharmacological inhibition, respectively. As shown in Figure 3A, transfection with an siRNA construct targeting SphK1, which reduced its mRNA and protein levels by 75-90% (shown in Figure 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 (Figure 3B). Taken together these data indicate that SphK1 expression and activity are required for IGFBP-3 to enhance EGFR signaling in TNBC cells.
SKi-II 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 μM) 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%) (Figure 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 (Figure 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 Figure 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 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 co-operatively 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 Figure 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 Figure S3B).

To study the effects of the EGFR kinase and SphK1 inhibitors in greater 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 using the CyQuant assay. Cells were sparsely plated in 96-well plates, changed to medium containing inhibitors 16 h later, and proliferation over the following 72-140 h was imaged using an IncuCyte apparatus. As shown in Figure 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 h onwards) and MDA-MB-436 (at the latest time point, 128 h). Remarkably in view of these 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 h for Hs578T, 52 h for MDA-MB-468, 56 h for MDA-MB-231 and 68 h 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 Figure 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 (Figure 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 μM) of gefitinib (P<0.05). The four cell lines showed differing patterns of activation of ERK1/2 and AKT in response to EGF (Figure 5B). In MDA-MB-231, EGF stimulated phosphorylation of AKT with very little 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 (Figure 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 3-6 similar experiments is given in Supplementary Figure 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 Figure 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 co-targeting 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 i.p. 3 times weekly. Tumor volumes were measured immediately before treatment was initiated, 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 mm$^3$). Prior to starting treatment, mice were randomized into treatment groups of 9-10 mice/group. The mean±S.E.M. of tumor volumes in the groups prior to treatment were: control, 146.1±20.2 mm$^3$; gefitinib, 138.5±18.6 mm$^3$; SKi-II, 151.6±20.7 mm$^3$; combination, 142.1±21.5 mm$^3$ (P=NS). As shown in Figure 6A, which depicts the mean±S.E.M. 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 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) or gefitinib (P=0.82) alone significantly inhibited tumor growth compared to 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±S.E.M. of tumor volumes of the combination group was 243.3±22.84 mm$^3$, compared with control of 398.1±48.24 mm$^3$ (P<0.05). The tumor volumes of the gefitinib (331.8±49.7 mm$^3$) and SKi-II (309.7±35.4 mm$^3$) groups did not differ significantly from control.

Western analysis of activation of EGFR, AKT and MAPK in excised tumors (Figure 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 compared with gefitinib alone, this did not reach statistical significance (P=0.06).
DISCUSSION.

This study sought to investigate the potential efficacy of co-targeting growth-stimulatory signaling pathways of two proteins that are highly expressed in TNBC: EGFR and IGFBP-3. Since 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 ER-ve breast tumors and cancer cells compared to ER+ve (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 since 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 sphingosine 1-phosphate (S1P) (37). While 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, ranging in size from ~42 kDa 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 biological 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 towards the accumulation of pro-apoptotic 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 where 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 ER+ve breast cancer cells (19), which would be another reason to target the SphK1 pathway rather than IGFBP-3 itself in TNBC where 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 where neither gefitinib or 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 pharmacological SphK inhibitors are being evaluated in pre-clinical
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 SphK1 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 pre-clinical 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 triple-negative breast cancer that could be rapidly implemented in the clinic.
REFERENCES


**FIGURE LEGENDS**

**Figure 1. Protein expression and activation in breast cancer cell lines.**

*A.* IGFBP-3 mRNA expression (upper panel) and secreted protein levels in 24 h conditioned medium (lower panel) from cells maintained in 5% FBS were measured using qRT-PCR and RIA, respectively, as described in the Materials and Methods. IGFBP-3 mRNA expression in each line is expressed relative to that in ZR-75-1 cells, which had the lowest level of IGFBP-3 expression (arbitrarily set as 1).

*B.* Confluent cultures of breast cancer cells were incubated for 24 h in medium containing 5% FBS. Conditioned media were collected for Western analysis of secreted IGFBP-3, and cells were lysed in Laemmli buffer. Lysates and media were analyzed using 7.5% SDS-PAGE and Western blotting using the indicated antibodies (as specified in the Materials and Methods), with α-tubulin as a loading control. Replicate blots were used for phosphorylated and total proteins. Migration points of molecular mass markers are shown on the right of each blot. The white, black and gray arrows shown to the left of the SphK1 immunoblot indicate SphK1a (42 kDa), SphK1b (51 kDa), and SphK1c (44 kDa) respectively, determined in TNBC cells as shown in Panel C.

*C.* qRT-PCR (upper panels) and Western analysis (lower panels) of SphK1 gene and protein expression in TNBC cell lines after siRNA-mediated SphK1 silencing as described in Materials and Methods, using either of two SphK1 siRNA (SK#1 and SK#2), or non-silencing siRNA (ctl). The level of SphK1 mRNA for each cell line is expressed relative to non-silencing control siRNA. For Westerns, the migration of molecular mass standards is shown on the left, and the white, black and gray arrows indicate SphK1a (42 kDa), SphK1b (51 kDa), and SphK1c (44 kDa) respectively. Two predominant non-specific bands which were not decreased when SphK1 was silenced (of ~45 and 53 kDa) are indicated by asterisks to the left of each panel. All blots have been cropped to highlight relevant bands.
Figure 2. Endogenous and exogenous IGFBP-3 enhance EGFR activation in TNBC cell lines.

A. Cells were incubated with the indicated concentration of IGFBP-3 for 16 h, then stimulated with EGF (10 ng/mL final concentration) for 10 min, lysed in Laemmli buffer, and analyzed by Western blotting for phosphorylated EGFR (Y1068) and total EGFR, with \( \alpha \)-tubulin as a loading control. Quantification of pooled data from 4 experiments is shown as mean±S.E.M. with 100% representing EGF-stimulated EGFR phosphorylation in the absence of IGFBP-3. Bars having different letters are significantly different from each other (P<0.05). B. Cells were treated with 100 ng/mL recombinant IGFBP-3 for 0, 30, 60 or 120 minutes, as indicated. Membranes were isolated as described in the Materials and Methods, and subjected to Western blotting to detect SphK1 and aquaporin 1 (AQP1), a plasma membrane marker. A representative blot of 3 for each cell line is shown. The histogram depicts quantified data as mean±S.E.M. from 3 experiments and indicates the calculated ratio of SphK1 to AQP1, determined by densitometric analysis of immunoblots. Bars with different letters are significantly different from each other (P<0.05). Uncropped images are shown in Supplementary Figure S2. C. Endogenous IGFBP-3 expression was reduced by transfection with either of two siRNA constructs (BP#1 and BP#2) or non-silencing control (ctl). Two days later, cells were stimulated with EGF (10 ng/mL) for 10 min, then harvested into Laemmli buffer for analysis of phosphorylated EGFR (Y1068) and total EGFR by Western blotting, with \( \alpha \)-tubulin as loading control. EGFR phosphorylation without EGF stimulation is also shown for MDA-MB-468 cells transfected with control or IGFBP-3 siRNA. Blots are representative of 3 experiments. Quantification of pooled data for BP#1 siRNA from 3 experiments is shown as mean±S.E.M. with 100% representing EGF-stimulated EGFR phosphorylation in ctl siRNA samples. All blots have been cropped to highlight relevant bands. Migration points of molecular mass markers are shown on the right of each blot.
Figure 3. Potentiation of EGFR activation by IGFBP-3 requires SphK1.

A. One day after non-silencing control (ctl) or SphK1 siRNA transfection, IGFBP-3 was added and incubations were continued for a further 16 h. Cells were stimulated with EGF (10 ng/mL) for 10 min, then harvested into Laemmlli buffer for analysis of phosphorylated and total EGFR by Western blotting, with α-tubulin as loading control. Graphs below each blot show quantification of EGFR phosphorylation relative to that stimulated by EGF in the absence of exogenous IGFBP-3, with data derived from 3 experiments (mean±S.E.M). B. Cells were incubated in medium without or with 10 μM SKi-II for 4 h, then IGFBP-3 was added to final concentrations of 10 or 100 ng/mL, as indicated, for 16 h. EGF was added (10 ng/mL final), incubations were continued for 10 min, then cells were harvested into Laemmlli buffer for analysis of phosphorylated EGFR (Y1068) and total EGFR by Western blot. Representative blots of 3 in total are shown, and the data depicted in histograms are mean±S.E.M derived from the pooled results for IGFBP-3 at 100 ng/mL in 3 experiments. All blots have been cropped to highlight relevant bands. Migration points of molecular mass markers are shown on the right of each blot.

Figure 4. SKi-II induces degradation of SphK1 in TNBC cell lines.

A. Cells were incubated with SKi-II (0, 1, 3 and 10 μM) without or with 10 μM gefitinib for 24 h. Harvested lysates were analyzed for SphK1 expression by Western blot, with the band representing SphK1a (42 kDa) shown. One representative blot of 3 is shown. Bar graphs in the lower panel depict quantification of data for gefitinib (10 μM) and SKi-II (10 μM) used alone and in combination. B. Cells were incubated for 24 h with SKi-II (10 μM) in the absence or presence of the proteasome inhibitor MG132 (1 μM), and lysates were analyzed for SphK1a. For A and B, densitometric quantification of the SphK1a data is expressed as a percentage of control (no gefitinib, SKi-II or MG132). Quantification of pooled data from 3 experiments is shown as mean±S.E.M, with bars having different letters being significantly different from
each other (P<0.05 by ANOVA). All blots have been cropped to highlight relevant bands. Migration points of molecular mass markers are shown on the right of each blot.

**Figure 5. Effect of combined gefitinib and SKi-II on TNBC cell proliferation and activation of signaling intermediates.**

_A_. TNBC cells were plated in 96 well plates in complete medium, then changed 24 h later to medium containing 5% FBS alone (●), or with SKi-II (□), gefitinib (■) or a combination of the two (○). The concentrations of inhibitors used were: MDA-MB-231, 1 μM gefitinib, 1 μM SKi-II; MDA-MB-436, 2 μM gefitinib, 1 μM SKi-II; MDA-MB-468, 1 μM gefitinib, 0.3 μM SKi-II; Hs578T, 4 μM gefitinib, 2.5 μM SKi-II. Cell proliferation was measured in real time over 3-5 days using an IncuCyte apparatus, as described in the Materials and Methods. ns, not significant at final time point; *, P<0.05 and **, P<0.001 at final time point, by repeated measures ANOVA. _B_. Cells were incubated with the indicated concentration of gefitinib in the absence or presence of 10 μM SKi-II for 4 h, then stimulated with EGF (10 ng/mL final) for 10 min. Harvested lysates were analyzed by Western blot for phosphorylated EGFR (Y1068), AKT (S473), and ERK1/2 (T202/Y204) on single filters probed sequentially for these proteins. A replicate filter was probed with antibodies to detect total proteins. The first lane for each cell line contains samples that have not been stimulated with EGF, and blots shown are representative of 3-6 experiments. All blots have been cropped to highlight relevant bands. Migration points of molecular mass markers are shown on the right of each blot.

**Figure 6. Growth of xenograft MDA-MB-468 tumors is inhibited by combined blockade of SphK1 and EGFR pathways.**

Tumors were established from MDA-MB-468 cells in female athymic BALB/c-Foxn1nu mice as described in the Materials and Methods, then animals were randomly allocated to groups of 9-10 for treatment. Gefitinib (75 mg/kg), SKi-II (50 mg/kg), combination, or vehicle control
(50 μL DMSO) were administered i.p. 3 times weekly, then animals were euthanized and tumors collected. **A.** Change in tumor volume from day 0 (first day of treatment) to days 5 and 11 was calculated for each animal, and data were pooled for each treatment. Shown are mean±S.E.M. with significance determined by repeated measures ANOVA and Bonferroni post-hoc test. **B.** Western analysis of EGFR, AKT and ERK1/2 in excised tumors (3/treatment). Tumor lysates were prepared as described in Material and Methods, and subjected to Western blotting for total and phosphorylated EGFR, AKT and ERK1/2. Bar graphs show quantified data (mean±S.E.M.) from 5 tumors for each treatment analyzed in the same way, with the effect of each treatment expressed relative to control. Bars in each graph having different letters are significantly different from each other (P<0.05 by ANOVA). All blots have been cropped to highlight relevant bands. Migration points of molecular mass markers are shown on the right of each blot.
Figure 2.

A

B

C
Figure 3.
Figure 4.

A

**MDA-MB-231**

- SphK1a
- α-tubulin
- SKI-II

0 gefitinib, 10 μM

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**MDA-MB-468**

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**Hs578T**

- 40 kDa
- 55 kDa

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B

**MDA-MB-231**

- SphK1a
- α-tubulin

ctl SKI MG SKI + MG

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**MDA-MB-436**

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**MDA-MB-468**

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**Hs578T**

- 40 kDa
- 55 kDa

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**Figure 5.**

(A) Confluence (%) over time (h) for MDA-MB-231, MDA-MB-436, MDA-MB-468, and Hs578T cell lines. Each graph shows the effect of gefitinib at various concentrations on cell confluence, with **ns** indicating no significant difference and **p** values indicating statistical significance.

(B) Western blot analysis for pEGFR, EGFR, pAKT, AKT, pERK1/2, and ERK1/2 in MDA-MB-231, MDA-MB-436, MDA-MB-468, and Hs578T cell lines treated with gefitinib at different concentrations (0, 0.25, 1, 5, 10 μM) and SKI-II (0, 5 μM) for MDA-MB-231 and MDA-MB-436, and SKI-II (0, 10 μM) for MDA-MB-468 and Hs578T.
Figure 6.

A

change in tumor vol, mm$^3$

0 50 100 150 200 250 300

day 0 day 5 day 11

vehicle gefitinib SKi-II combination

ns $P = 0.011$

B

pEGFR EGFR

pAKT AKT

pERK1/2 ERK1/2

ctl gefitinib SKi-II combination

pH phospho/total

ctl gef SKi-II comb

ctl gef SKi-II comb

ctl gef SKi-II comb

ctl gef SKi-II comb
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.

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