SPHINGOSINE 1-PHOSPHATE RECEPTOR 4 USES HER2 (ERBB2) TO REGULATE EXTRACELLULAR SIGNAL REGULATED KINASE-1/2 IN MDA-MB-453 BREAST CANCER CELLS

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We demonstrate here that the bioactive lipid sphingosine 1-phosphate (S1P) uses the sphingosine 1-phosphate receptor 4 (S1P4) and human epidermal growth factor receptor 2 (HER2) to stimulate the extracellular signal regulated protein kinase 1/2 (ERK-1/2) pathway in MDA-MB-453 cells. This was based on several lines of evidence. First, the S1P stimulation of ERK-1/2 was abolished by JTE013, which we show here is an S1P2/4 antagonist and reduced by siRNA knock down of S1P4. Second, the S1P-stimulated activation of ERK-1/2 was almost completely abolished by a HER2 inhibitor (ErbB2 inhibitor II) and reduced by siRNA knock down of HER2 expression. Third, phyto-S1P, which is an S1P4 agonist, stimulated ERK-1/2 activation in an S1P4- and HER2-dependent manner. Fourth, FTY720 phosphate which is an agonist at S1P1, 3, 4, 5 but not S1P2 stimulated activation of ERK-1/2. Fifth, S1P stimulated the tyrosine phosphorylation of HER2, which was reduced by JTE013. HER2 which is an orphan receptor tyrosine kinase is the preferred dimerisation partner of the EGF receptor. However, EGF-stimulated activation of ERK-1/2 was not affected by siRNA knock down of HER2 or by ErbB2 inhibitor II in MDA-MB-453 cells. Moreover, S1P-stimulated activation of ERK-1/2 does not require EGF receptor. Thus, S1P and EGF function in a mutually exclusive manner. In conclusion, the magnitude of the signaling gain on the ERK-1/2 pathway produced in response to S1P can be increased by HER2 in MDA-MB-453 cells. The linkage of S1P with an oncogene suggests that S1P and specifically S1P4 may have an important role in breast cancer progression.

There is increasing evidence to suggest a role for the bioactive lipid sphingosine 1-phosphate (S1P) in breast cancer. S1P binds to a family of five GPCR termed S1Pn (where n=1-5), that are differentially coupled to heterotrimeric G-proteins (G\textsubscript{i}, G\textsubscript{q} and G\textsubscript{12/13}) to regulate various effectors such as MAP kinases linked to diverse cellular processes such as proliferation, cell survival and differentiation (1-13). The specificity of signaling in terms of which kinase modules are activated is dependent upon the receptor sub-type involved and is cell context specific. S1P is produced by the enzyme sphingosine kinase (SK), which catalyses the phosphorylation of sphingosine to produce S1P. SK (which exists as two isoforms termed SK1 and SK2) is activated by agonists such as antigen, platelet-derived growth factor, nerve growth factor, tumour necrosis factor alpha and epidermal growth factor (EGF) resulting in an increase in intracellular S1P. SK1 activation and/or translocation is regulated by phosphorylation catalysed by ERK-1/2 and by calcium and integrin-binding protein 1 (CIB1) (14, 15).

S1P has an important role in breast cancer cells in terms of regulating survival, proliferation and migration (16-18). For instance, ectopic expression of SK1 increased S1P levels, estrogen-dependent...
tumourigenesis, and blocked apoptosis of MCF-7 cells induced by anti-cancer drugs, sphingosine and tumour necrosis factor alpha (16). SK1 and S1P are also required for EGF-induced MCF-7 migration, proliferation and cell survival (18). S1P also stimulates breast cancer cell growth through activation of the serum response element and indirectly by enhancing IGF-II synthesis and function (19).

The HER2/neu/c-erbB-2 gene encodes a 185 kDa transmembrane receptor tyrosine kinase, which is related to other members of the EGF receptor family (20). Moreover, the over-expression of HER2/neu is found in up to 30% of primary breast cancers and increased tumour invasion, poor prognosis and therapeutic resistance is correlated with its expression (21). A soluble ligand for HER2 has not been identified, although HER2 operates as a shared receptor subunit of other ErbBs. In this regard, HER2 is a heterodimerisation partner of the EGF receptor (22). HER2 delays EGF dissociation from its receptor, improves coupling of EGF receptor and stimulation of the ERK-1/2 pathway, and impedes EGF receptor down-regulation. Thus, HER2 is a master regulator that drives epithelial cell proliferation. An example of this is evident from studies which demonstrate that the ectopic expression of HER2/neu in MCF-7 (estrogen receptor (ER) positive/HER2 negative) cells stimulates the PI3K/Akt pathway and down regulates p53 (23), which increases the cell survival.

Breast cancer cell lines can be categorised into three major phenotypic groups that include: (i) luminal epithelial like ER positive/HER2 negative cells (e.g. MCF-7 cells, weakly invasive); (ii) weakly luminal epithelial like HER2 positive cells (e.g. MDA-MB-453 cells); (iii) stromal/mesenchymal phenotype (e.g. MDA-MB-231), which are characterised as ER negative/HER2 negative cells.

In this study, we have used MDA-MB-453 cells and HER2+/ER- breast cancer tumour samples in order to investigate the role of S1P in regulating the ERK-1/2 pathway, which is well established as having a role in cancer metastasis.

Experimental Procedures

Materials—All general biochemicals were from Sigma-Aldrich (Poole, UK). High glucose Dulbecco's modified Eagle's medium and European Foetal Calf Serum, penicillin-streptomycin were from Invitrogen (Paisley, UK). DharmaFECT™ 2 reagent was from Dharmacon (Dharmacon, Cromlington, UK). BioScript™ was from Bioline (London, UK). HER2 and S1P2 and S1P4 siRNA and anti-phosphorylated ERK1/2 antibody were from Santa Cruz (California, USA). EGF receptor siRNA was a gift from V. Natarajan (University of Chicago, USA). Anti-ERK2 and anti-HRP tyrosine phosphate antibodies were from BD Transduction Laboratories (Oxford, UK). Anti-HER2 antibody was from New England Biolabs (UK) Ltd (Hitchin, UK). S1P and phyto-S1P were from Avanti Polar Lipids (Alabaster, USA), EGF from Sigma (Poole, UK), JTE013 from Tocris Biosciences (Bristol, UK) and CAY10444 and SEW2871 from Cayman Chemicals (Tallinn, Estonia). Recombinant Heregulin, ErbB2 inhibitor II, AG 879 and AG 1478 were from Merck Biosciences (Nottingham, UK). FTY720 and FTY720 phosphate were gifts from R. Bittman (CUNY, USA).

Cell Culture—MDA-MB-231 and MDA-MB-453 breast cancer cell lines were obtained from the ATCC (Rockville, MD) and were grown in a monolayer culture in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% European Fetal Calf Serum (EFCS) and 1% Pen-Strep (Penicillin G Sodium 104 units/ml-Streptomycin Sulphate 10 mg/ml) at 37°C with 5% CO2. HEK 293 cells were grown as above with the exception that minimum essential medium (MEM) was used instead of DMEM. MCF-7 Neo and MCF-7 HER2 breast cancer cells (from R. Schiff, Baylor College, USA) were grown in a monolayer culture in DMEM with 10% EFCS and 1% Pen-Strep, 0.4% Geneticin and 15μg/ml Insulin at 37°C with 5% CO2. HTC4 cells stably expressing S1P2, S1P3, or S1P4 receptors were maintained in DMEM containing 10% Fetal Bovine Serum and 500μg/ml of geneticin.

siRNA Treatment—Knock down of HER2 expression was achieved using sequence-specific HER2 siRNA (HER2 receptor siRNA (AAGGGGCUGGCUCCGAGUUAUUdTDt and AAUACAUCCAGCCGCGCCCUCUdTdT)) and scrambled siRNA as control (GCUGACGAGUGGCAUCUUAAGUdTDt and ACAUUAAGACAGCCGACCAGdTdT). The HER2 receptor targeted sequence is AAGGGGCUCCGAGUUAUUU.

siRNA transfection was performed according to the protocol detailed by Dharmacon. Briefly, cells grown on 24-well plates were transfected with 100-400 nM siRNA prepared in a mix with DharmaFECT™ 2 reagent and DMEM containing 10% EFCS. Cells were also treated with EGF receptor
siRNA (100 nM, Dharmacon) in the same manner. The cells were cultured for 48 hours before being serum-starved for 24 hours prior to stimulation. *RNA Extraction and Real-Time Quantitative RT-PCR of S1P Receptor mRNA*—RNA was isolated from MDA-MB-453 cells using TRIzol (Invitrogen, Carlsbad, CA). cDNA synthesis was performed by using the SuperScript First Strand Synthesis kit (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Amplification was performed for 40 cycles at 94 °C for 30 seconds, 50 °C for 60 seconds, and 72 °C for 60 seconds with initial activation of enzyme at 95 °C for 1 minute using ABI Model 7300 Real Time PCR machine. The following primer pairs were used: GAPDH (forward) CTGCAGGTCATCTCCCTCAT and (reverse) GGCTGGTCATCATCCCTCAT and S1P1 (forward) GGTGAGCGAGGTCATCGT and (reverse) CCAGGAGCAGGAACATGG. S1P2, S1P3, and S1P4 primers were designed by us (24) using anti-phosphorylated ERK1/2, anti-HER2 and anti-HRP antibodies.

Quantitative values were obtained from the threshold cycle value (Ct). GAPDH was quantified as an internal RNA control, and each sample was normalized on the basis of its GAPDH content. Samples were run in quadruplicate. A Student’s t-test at a P value of 0.05. Data were plotted and fitted to a sigmoid function by using the nonlinear curve-fitting feature of KaleidaGraph (Synergy Software, Essex Junction, VT).

**Immunoprecipitation**—The medium was removed and cells lysed in ice-cold immunoprecipitation buffer (1 ml) containing 20 mM TRIS/HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 1% (v/v) Nonidet P-40 (NP-40), 10% (v/v) glycerol, 1 mg/ml bovine serum albumin, 0.5 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, leupeptin and aprotinin (all protease inhibitors were at 10 µg/ml) at pH 8 for 10 min at 4°C. The material was harvested, centrifuged at 22 000 g for 5 min at 4°C and 200-400 µL of cell lysate supernatant (equalized for protein, 0.5-1 mg/ml) combined with 20 µl of one part immunoprecipitation buffer and one part protein A or G Sepharose and incubated for 20 min at 4°C. The samples were centrifuged at 22 000 g and the supernatant taken for immunoprecipitation with anti-HER2 or anti-ERK-1/2 antibody (5 µg of anti-HER2 antibody or 3 µg of anti-ERK-1/2 antibody and 20-40 µl of one part immunoprecipitation buffer and one part protein A or G Sepharose CL4B respectively). After agitation for 2 h at 4°C, the immune complex was washed with 1 ml of salt buffer (150 mM NaCl in modified Krebs buffer containing 2% (v/v) pluronic acid. After incubating the cells with Fura-2 AM, the cells were rinsed with Krebs buffer and changes in the intracellular Ca2+ concentration were monitored by determining the ratio of emitted light intensities at 520 nm in response to excitation at 340 and 380 nm using FLEXstation II (Molecular Devices, Sunnyvale, CA). Each well was monitored for 80 seconds. To test antagonist activity of the inhibitors, JTE013 and CAY10444, increasing concentrations of the inhibitors were mixed with a constant concentration of S1P, and added automatically after 15 seconds of baseline measurement. Each test was performed in quadruplicate. Significant difference between two experimental groups was determined by the Student’s t-test at a P value of 0.05. Data were plotted and fitted to a sigmoid function by using the nonlinear curve-fitting feature of KaleidaGraph (Synergy Software, Essex Junction, VT).
collected by centrifugation at 22 000 g for 15 s at 4°C. Immunoprecipitates were washed twice with buffer A containing 10 mM HEPES, pH 7, 100 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 µg/ml aprotinin and 0.5% (v/v) NP-40 and once in buffer A without NP-40. The immunoprecipitates were then combined with boiling sample buffer containing 62 mM TRIS HCl, pH 6.7, 1.25% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 3.75% (v/v) mercaptoethanol and 0.05% (w/v) bromophenol blue. The samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting.

MBP Kinase Assay- Immunoprecipitates were combined with a phosphorylation cocktail containing 20mM Hepes pH 7, 5mM-MgCl₂, 25µM ATP and 5µCi [γ-32P]ATP and 1µg Myelin Basic Protein and incubated at 30°C for 10 min. Incubations were terminated by addition of boiling sample buffer and subjected to SDS-PAGE.

Immunohistochemistry- The cell pellet slides were first dewaxed and rehydrated through a series of xylene and alcohol washes. Antigen retrieval was performed for S1P2 by microwaving the slides under pressure in a TRIS EDTA buffer for 5 minutes (pH 8.0) and for S1P4 in 10mM Citrate Buffer at 96°C in for 20 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxide for 20 minutes and non-specific background staining was reduced by blocking with a 1:20 concentration of horse serum diluted in TRIS buffered saline for 30 minutes. The sections were incubated with the primary antibody for S1P2 and S1P4 (Exalpba, Shirley, MA, USA). Each antibody was incubated at a dilution of 1:100 at 4°C overnight. EnVision-HRP conjugate (DAKO, Cambridgeshire, UK) was used for signal amplification and positive staining was identified using 3, 3'-diaminobenzidine (DAB) chromagen (Vector Laboratories). The slides were then counterstained with haematoxylin and Scott’s Tap Water Substitute before dehydration and mounting.

Densitometry- Densitometric quantification of western blots was performed using the Molecular Analyst Software (Bio-Rad Laboratories Ltd). Statistical analysis was performed with the GraphPad Prism software, using one-way ANOVA followed by Newman-Keuls post-hoc test.

RESULTS

Regulation of ERK-1/2 by S1P in MDA-MB-453 cells. MDA-MB-453 cells are HER2+ as evidenced by immunodetection of HER2 (Mr=185kDa) with anti-HER2 antibody (Fig 1a). In these experiments MDA-MB-231 cells were used as a negative control for western blotting, as these cells are HER2 null (Fig. 1a). Quantitative real time PCR revealed that S1P3 was the most abundant S1P receptor mRNA transcript, with small quantities of S1P2,4 and very minor expression of S1P1/5 mRNA (Fig. 1b).

Treatment of MDA-MB-453 cells with S1P stimulated the phosphorylation/activation of ERK-1/2, which was reduced by pre-treating these cells with pertussis toxin (PTX, which uncouples GPCR from G_i) (Fig 2a, b). These data suggest that S1P uses a heterotrimeric G-protein coupled receptor to regulate the ERK-1/2 pathway. PTX did not significantly reduce EGF-induced activation of ERK-1/2 (Fig. 2a, b, P>0.05 for EGF + PTX versus EGF). The identity of the S1P receptor involved in regulating the ERK-1/2 pathway was evaluated using pharmacological agents that demonstrate selectivity at S1P receptors. We used JTE013, which is reported to be an S1P2 selective antagonist (28), and CAY10444 (29), which is an S1P3 antagonist.

To further characterise the pharmacological specificity of JTE013 and CAY10444 we used HTC4 cells in which S1P2, 3, 4 were separately stably expressed and intracellular calcium mobilisation was measured using the Fura-2 indicator dye. We found that JTE013 reduced S1P-stimulated calcium mobilisation in cells over-expressing S1P2, while CAY10444 was very weakly effective at concentrations >1µM (Fig. 3a). In contrast JTE013 had no effect on calcium mobilisation induced by S1P in cells over-expressing S1P3, while CAY10444 was very weakly effective at concentrations >1µM (Fig. 3a). In contrast JTE013 had no effect on calcium mobilisation induced by S1P in cells over-expressing S1P3, while CAY10444 reduced this response (Fig. 3a), thereby confirming specificity of CAY10444 at S1P3. Surprisingly, we found that JTE013 can also function as an S1P4 antagonist, as evidenced by results showing that JTE013 potently reduced S1P-stimulated calcium mobilisation (Ki=236.9 nM) in cells over-expressing S1P4, while CAY10444 was without effect (Fig. 3a). As there are no selective S1P4 antagonists available, we used JTE013 as a tool to investigate the role of S1P2,4 in mediating the effect of S1P on the activation of ERK-1/2 in MDA-MB-453 cells. We found that the S1P-induced activation of ERK-1/2 was substantially reduced by JTE013 and was not affected by CAY10444 (Fig. 3b, c).
453 cells lack functional S1P₁ was evidenced by the finding that the S1P₁ selective agonist SEW2871 was without effect on ERK-1/2 activation (Fig. 3d).

We also used specific siRNA approaches to knock down expression of the S1P₄ receptor in MDA-MB-453 cells. In this regard, we found that siRNA knock down of S1P₄ reduced the S1P₄-stimulated activation of ERK-1/2 (Fig. 4a, b). We confirmed by QRT-PCR that S1P₄ siRNA reduced S1P₄ mRNA transcript and had no effect on S1P₂ or S1P₃ mRNA transcript (Fig. 4c). We also detected S1P₄ protein (M=42kDa) in lysates of MDA-MB-453 cells on western blots probed with anti-S1P₄ antibody and found that S1P₄ siRNA partially reduced expression (55 ± 12% reduction, n=5) of the protein (Fig. 4c). In addition, IHC of MDA-MB-453 cells with anti-S1P₄ antibody demonstrated that siRNA knock down of S1P₄ reduced immunostaining (Fig. 4d). We could also confirm increased immunostaining when MDA-MB-453 cells were transfected with S1P₄ plasmid construct (Fig. 4d). Collectively, these data confirm expression of S1P₄ protein in MDA-MB-453 cells, specificity of the antibody and effectiveness of the S1P₄ siRNA treatment.

Evidence to exclude S1P₂ was obtained using S1P₂ siRNA, which did not reduce activation of ERK-1/2 by S1P (Fig. 4e). IHC of MDA-MB-453 cells with anti-S1P₂ antibody demonstrated that siRNA knock down of S1P₂ eliminated immunostaining (Fig. 4d), thereby confirming expression of S1P₂ protein in MDA-MB-453 cells, specificity of the antibody and successful knockdown of S1P₂. Knock down of S1P₂ with specific siRNA was more effective than corresponding knock down of S1P₁ with its respective siRNA (Fig. 4d). The residual S1P₂ expression after siRNA knock down might explain the incomplete abolition of S1P₂-stimulated ERK-1/2 activation with S1P₂ siRNA (Fig. 4a) while JTE013 via S1P₄ antagonism is completely effective (Fig. 3b).

In addition, we used FTY720 (which is phosphorylated by SK2 to FTY720 phosphate) and FTY720 phosphate, which is an agonist at S1P₁, 3, 4, 5 but not S1P₂, and which stimulated activation of ERK-1/2 in MDA-MB-453 cells (Fig. 4f). Finally, we used phyto-S1P, which is an agonist at S1P₄ (30) and demonstrated that this agent also induced activation of ERK-1/2 (Fig. 4g), which was reduced by siRNA knockdown of S1P₄ (Fig. 4g), but not S1P₂ (Fig. 4e). S1P₄ knock down was more effective against phyto-S1P compared with S1P. This apparent anomaly might be explained by a model in which there is higher fractional receptor occupancy with phyto-S1P compared with S1P for ERK-1/2 activation, and where efficacy for each phyto-S1P bound S1P₄ receptor is less than for each S1P bound S1P₄ receptor. We conclude that S1P₄ mediates the effect of S1P on ERK-1/2 activation in MDA-MB-453 cells.

**SIP Receptor Functional Interaction with HER2.** Evidence that a functional interaction occurs between S1P and HER2 was obtained by the finding that S1P, but not EGF, stimulated the tyrosine phosphorylation of HER2 and this was severely reduced by JTE013 (Fig. 5a, b). Evidence for the involvement of HER2 in regulating S1P₄ signaling to the ERK-1/2 pathway was demonstrated by results showing that siRNA knock down HER2 expression reduced S1P-stimulation of ERK-1/2 by ~ 50% compared to cells treated with scrambled siRNA (Fig. 6a, b). The incomplete reduction in ERK-1/2 activation might be due to residual HER2 expression after siRNA treatment. To confirm data obtained by western blotting with anti-phospho ERK-1/2 antibody, we immunoprecipitated ERK-1/2 with anti-HER2 antibody and measured ERK-1/2 activity against MBP in the immunoprecipitates. This assay confirmed that S1P or EGF stimulated ERK-1/2 activity (Fig. 6a). Moreover, siRNA knock down of HER2 reduced the stimulation of ERK-1/2 activity by S1P and had no effect on the response to EGF (Fig. 6a). HER2 siRNA had no effect on cell integrity as assessed using an MTT assay (Fig. 6a). We also used the HER2 inhibitor, ErbB2 inhibitor II (4-(3-Phenoxyphenyl)-5-cyano-2H-1,2,3 - triazole), which is a cell permeable HER2 ATP binding kinase inhibitor and reduces phosphorylation of HER2 in MDA-MB-453 cells but not that of over-expressed EGFR in MDA-MB-468 cells, even at concentrations as high as 100µM (31). The inhibitor was discovered from a computer aided drug design approach and searched from molecule libraries. Modelling has also been used to demonstrate binding of the inhibitor in the HER2 ATP binding site (31).

We found that the pre-treatment of MDA-MB-453 cells with the ErbB2 inhibitor II reduced basal ERK-1/2 phosphorylation (Fig. 6c) suggesting a tonic influence of HER2 on this pathway. However, this does not explain the involvement of HER2 in S1P₄ receptor signaling, as S1P-stimulated ERK-1/2 activation was almost completely abolished by ErbB2 inhibitor II while the response to EGF was unaffected (Fig. 6c). The ability of ErbB2 inhibitor II to reduce basal ERK-1/2 activation differs from HER2 siRNA, which had no significant effect (Fig. 6a, b). These
findings suggest that the knockdown of HER2 with siRNA, which is incomplete, might not be sufficient to ablate basal ERK-1/2 activation, but is able to reduce S1P signaling via S1P_4. Thus, basal and S1P-stimulated ERK-1/2 activation may have different requirements for HER2 e.g. less HER2 is required to sustain the basal ERK-1/2 activation compared with the S1P-stimulated activation of ERK-1/2.

In addition, phyto-S1P-stimulated activation of ERK-1/2 was also reduced by siRNA knock down of HER2 (Fig. 6d). We have therefore demonstrated that S1P binding to S1P_4 engages HER2 to regulate the ERK-1/2 pathway in MDA-MB-453 cells.

**EGF Regulation of ERK-1/2 in MDA-MB-453 and MCF-7 Cells.** HER2 is an orphan receptor tyrosine kinase and is the preferred dimerisation partner of the EGF receptor. However, we have demonstrated here that the EGF-induced activation of ERK-1/2 was not reduced by the siRNA knock down of HER2 (Fig. 6a, b). Thus, S1P and EGF function in a mutually exclusive manner. In addition, S1P does not use the EGF receptor tyrosine kinase to regulate ERK-1/2. Thus, the activation of ERK-1/2 by EGF was reduced by the EGF receptor tyrosine kinase inhibitor AG 1478 (Fig. 6a, b) and by siRNA knockdown of EGF receptor expression (Fig. 7c, d). However, the S1P-stimulated activation of ERK-1/2 was not modulated by AG 1478 (Fig. 7a, b) or by siRNA knock down of EGF receptor (Fig. 7c, d). Further evidence to support divergent signaling by S1P and EGF with respect to HER2 was the finding that unlike S1P, EGF did not induce the tyrosine phosphorylation of HER2 (Fig. 5a, b).

S1P_4 has no functional role in terms of regulating ERK-1/2 signaling in response to S1P in MDA-MB-453 cells (Fig. 3b). On the contrary S1P_4 signaling appears to predominate. We therefore asked the question: what is the molecular organisation of signaling from the S1P_4 receptor in other breast cancer cell types that lack S1P_4? For this purpose we used a breast cancer cell line, ER + MCF-7 cells, where S1P_3 mRNA is abundantly expressed but where S1P_4 mRNA is absent (Fig. 8a). Moreover, S1P-stimulated activation of ERK-1/2 is known to be mediated by the S1P_3 receptor and involves transactivation of the EGF receptor (S1P increases the tyrosine phosphorylation of the EGF receptor) in these cells (32). Indeed, we have confirmed that siRNA knockdown of S1P_3 or use of the S1P_3 antagonist, CAY10444, reduced the S1P-stimulated activation of ERK-1/2 in both MCF-7 Neo (express the Neo vector) and MCF-7 HER2 cells (HER218 cells, stably expressing HER2) by >90% (data not shown). We show here that the stimulation of ERK-1/2 by S1P in MCF-7 Neo and MCF-7 HER2 cells was reduced by pre-treating cells with AG 1478 (Fig. 8b, c). The higher basal ERK-1/2 activation in MCF-7 HER2 cells compared with MCF-7 Neo cells might be due to EGF receptor, as this was also reduced by AG 1478 (Fig. 8c). In conclusion, S1P/S1P_3 stimulation of ERK-1/2 is characterised by a requirement for EGF receptor tyrosine kinase activity in MCF-7 cells. In addition the S1P stimulation of ERK-1/2 in MCF-7 Neo (which lack HER2) and MCF-7 HER2 cells was unaffected by the HER2 kinase inhibitor, AG 879 (Fig. 8b, c).

We also tested whether heregulin might participate in S1P signaling in MDA-MB-453 cells. Interestingly, heregulin-stimulated activation of ERK-1/2 was reduced by AG 879 and AG 1478 in these cells (Fig. 9). However, the stimulation of ERK-1/2 by heregulin was insensitive to the S1P_3 antagonist, JTE013 and the S1P_3 antagonist, CAY10444 (Fig. 9).

**DISCUSSION**

We demonstrate here that S1P stimulates the ERK-1/2 pathway via a mechanism that involves HER2 and S1P_4 in MDA-MB-453 cells. This novel mechanism is based on several lines of evidence. First, we demonstrated that the S1P-induced activation of ERK-1/2 was reduced by JTE013, but not by CAY10444, an S1P_3 selective antagonist. We have shown here for the first time, that in addition to being an S1P_2 receptor antagonist, JTE013 is also a potent antagonist of S1P_4, and can block S1P-induced mobilisation of calcium in cells over-expressing this receptor. This is an important finding because JTE013 is used widely and is considered to have a high-degree of specificity for S1P_2. Second, siRNA knock down of S1P_4 reduced the activation of ERK-1/2 by S1P. Third, phyto-S1P, which is a selective S1P_4 agonist stimulated ERK-1/2 activation indicating that S1P_4 specific ligands are able to activate this kinase pathway in MDA-MB-453 cells. Moreover siRNA knock down of S1P_4 reduced the activation of ERK-1/2 by phyto- S1P. A role for S1P_4 in the regulation of ERK-1/2 is excluded based on results showing that: (i) siRNA knock down of S1P_4 had no effect on the stimulation of ERK-1/2 by either S1P or phyto-S1P; (ii) FTY720 (the phosphorylated equivalent of which does not bind to S1P_3) stimulated the activation of ERK-1/2. Three additional results provide evidence for a role of HER2 in regulating S1P_4 signaling in...
MDA-MB-453 cells. First, the S1P-stimulated activation of ERK-1/2 was almost completely abolished by treatment of cells with ErbB2 inhibitor II. Second, the S1P- and phyto-S1P-stimulated activation of ERK-1/2 was reduced by the siRNA knock down of HER2 expression. Third, S1P stimulated the tyrosine phosphorylation of HER2, which was reduced by JTE013.

We also found that S1P stimulation of ERK-1/2 via S1P4 in MDA-MB-453 cells does not involve participation of the EGF receptor, thereby excluding EGF release as a possible mechanism mediating the effects of S1P on this pathway. This contrasts with a role for EGF receptor down-stream of S1P3 in MCF-7 cells (this study and (32)). Regarding S1P3/ERK-1/2 signalling in MDA-MB-453 cells, the lack of participation of EGF receptor is based on the finding that the EGF receptor tyrosine kinase inhibitor, AG 1478 and siRNA knock down of EGF receptor expression reduced the EGF-dependent activation of ERK-1/2, but failed to modulate the response to S1P. Moreover, EGF receptor does not require HER2 to activate the ERK-1/2 pathway based on results showing that EGF-stimulated activation of ERK-1/2 was not reduced by ErbB2 inhibitor 2 or siRNA knock down of HER2. Furthermore, EGF failed to stimulate the tyrosine phosphorylation of HER2. Taken together, these findings demonstrate that S1P4 receptor is involved this process. The EGF receptor tyrosine kinase inhibitor, AG 1478 was shown to have no effect on the S1P-stimulated tyrosine phosphorylation of c-Met, thereby excluding EGF receptor as an intermediate between S1P3 and c-Met. Shida and colleagues also demonstrated that LPA and S1P induce the tyrosine phosphorylation of HER2 in MKN28 and MKN74 cells and this was dependent upon metalloproteinase-dependent release of EGF receptor ligands (36).

With respect to the studies described above our findings clearly describe a novel mechanism of HER2 transactivation and stimulation of the ERK-1/2 pathway in response to S1P in MDA-MB-453 cells. In this regard, we have previously demonstrated that GPCR and RTK can form functional signaling units that result in the RTK enhancing stimulation of the ERK-1/2 pathway by the respective GPCR ligand (24, 37, 38). For instance, we demonstrated that S1P1 and PDGFB receptor form a functional signaling unit, where the PDGFB receptor tyrosine kinase enhances S1P stimulation of ERK-1/2 mediated by S1P1 (24, 37). It is therefore possible that S1P4 and HER2 might form similar signaling units to regulate the ERK-1/2 pathway in MDA-MB-453 cells. In this case, the tyrosine phosphorylation of HER2 in response to S1P might produce a signaling platform that enables recruitment of regulatory/adaptor proteins via their SH2 interaction with phosphotyrosines on HER2 and which may facilitate stronger activation of the ERK-1/2 pathway in response to S1P. This possibility requires formal testing.

We have also shown that heregulin does not use either S1P3 or S1P4 to regulate the ERK-1/2 pathway. In addition, the potential release of heregulin in
response to S1P is unlikely because heregulin signaling is sensitive to AG 1478 and therefore requires EGF receptor, while S1P responses are insensitive to AG 1478.

In conclusion, we have demonstrated that the magnitude of the signaling gain on the ERK-1/2 pathway produced in response to S1P can be increased by an oncogene-HER2 and in an ER-breast cancer cell line. This is unusual as S1P4 expression and function is largely restricted to lymphoid cells such as T-cells (39). Therefore, S1P4 expression and function may exhibit some promiscuity in cancer cells. In addition, JTE013 can be considered a potent antagonist of S1P4, providing both a useful tool for interrogating the function of this receptor in breast cancer, and also as a prototype for further compound optimisation and translational approaches to target S1P4 in ER/HER2+ breast cancer.

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FIGURE LEGENDS

Fig._1 S1P receptors and HER2 in MDA-MB-453 cells. (a) Western blot of MDA-MB-453 and MDA-MB-231 cell lysates with anti-HER2 antibody; (b) bar chart showing relative mRNA expression of S1P1-5 in MDA-MB-453 cells, determined using QRT-PCR.

Fig._2 S1P signaling in MDA-MB-453 cells. MDA-MB-453 cells were pre-treated with PTX (0.1µg/ml) for 20 hours prior to stimulation with and without EGF (50ng/ml) or S1P (5µM) for 10 minutes. (a) The western blot shows the effect of PTX on S1P- and EGF-stimulated activation of ERK-1/2. Images are from the same western blot. Phosphorylated ERK-1/2 was detected on western blots probed with anti-phosphorylated ERK-1/2 antibody. Blots were also probed with anti-ERK-1/2 antibody to ensure equal protein loading; (b) bar chart quantifying the effect of PTX on ERK-1/2 activation. Results are expressed P-ERK-1/2:ERK-2 ratios for n=3 experiments.

Fig._3 S1P receptor specificity by JTE013 and CAY10444 and their effect in MDA-MB-453 cells. (a) Specificity of S1P antagonists: graphs showing the effect of JTE013 or CAY10444 on S1P-stimulated calcium mobilisation in HT-IC4 cells over-expressing recombinant S1P1 or S1P3 or S1P4; (b-d) MDA-MB-453 cells were pre-treated vehicle, JTE013 (10µM) or CAY10444 (10µM) for 15 minutes prior to stimulation with and without EGF (50ng/ml) or S1P (5µM or indicated concentration) or SEW 2871 (1 or 10µM) for 10 minutes. (b) The western blot shows the effect of JTE013 or CAY10444 on S1P- and EGF-stimulated activation of ERK-1/2 in MDA-MB-453 cells. The images for control/JTE013 with and without S1P/EGF and control/CAY10444 with and without S1P/EGF experiments were taken from the same western blots respectively; (c) bar chart showing the quantification of the effect of JTE013 and CAY10444 on S1P-EGF-stimulated activation of ERK-1/2 in MDA-MB-453 cells. The images for control/JTE013 with and without S1P/EGF and control/CAY10444 with and without S1P/EGF experiments were taken from the same western blots respectively; (d) western blot showing the lack of effect of SEW2781 on ERK-1/2 activation in MDA-MB-453 cells. In (b) and (d), phosphorylated ERK-1/2 was detected on western blots probed with anti-phosphorylated ERK-1/2 antibody. Blots were also probed with anti-ERK-1/2 antibody to ensure equal protein loading. In (c) results are expressed P-ERK-1/2:ERK-2 ratios for n=3 experiments.
Fig. 4 The effect of S1P, FTY720, FTY720 phosphate and phyto-S1P on the ERK-1/2 pathway and role of S1P$_{2/4}$ receptor in MDA-MB-453 cells. MDA-MB-453 cells were treated with scrambled siRNA or S1P$_4$ siRNA (200nM, 48 hours) or S1P$_2$ siRNA (100nM, 48 hours) prior to stimulation with and without S1P (1μM). Cells were also stimulated with FTY720 or FTY720 phosphate or phyto-S1P (at the indicated concentration) for 10 minutes. Western blots showing: (a) the effect of siRNA knock down of S1P$_4$ on S1P-stimulated activation of ERK-1/2; (b) bar chart showing quantification of the effect of S1P$_4$ siRNA on the S1P-induced activation of ERK-1/2; (c) quantification by QRT-PCR of S1P$_{2,3,4}$ mRNA expression in cells treated with scrambled or S1P$_4$ siRNA. Also shown is a western blot probed with anti-S1P$_4$ antibody to show siRNA knock down of S1P$_4$ in MDA-MB-453 cells (n=5 cell samples). HEK-HA-S1P$_4$ are samples from HA-S1P$_4$ over-expressing HEK 293 cell; (d) IHC staining showing the effect of S1P$_2$ siRNA and S1P$_4$ siRNA on the expression of S1P$_2$ and S1P$_4$ protein respectively in MDA-MB-453 cells. Cells were also co-stained with haematoxylin. AB denotes S1P$_2$ or S1P$_4$ antibody. Also shown is S1P$_4$ AB immunostaining of MDA-MB-453 cells transfected with S1P$_4$ plasmid construct; (e) the lack of effect of S1P$_2$ siRNA on S1P- or phyto-S1P (5μM)-stimulated activation of ERK-1/2; (f) the effect of FTY720 (5μM), (R)- and (S)-FTY720 phosphate (each at 5μM) on ERK-1/2 activation; (g) the effect of siRNA knock down of S1P$_4$ on phyto-S1P (5μM)-stimulated activation of ERK-1/2. Also included is a bar chart showing the quantification of the effect of S1P$_4$ siRNA on phyto-S1P-induced activation of ERK-1/2. Results in (b) and (g) are expressed as P-ERK-1/2:ERK-2 ratios for n=3 experiments. Results in (a), (e), (f) and (g) are representative of three separate experiments. Phosphorylated ERK-1/2 was detected on western blots probed with anti-phosphorylated ERK-1/2 antibody. ERK-2 was also detected with anti-ERK-2 antibody either on reprobes or in the same samples run on a separate SDS-PAGE to ensure comparable protein loading.

Fig. 5 S1P stimulates tyrosine phosphorylation of HER2. MDA-MB-453 cells were pre-treated with JTE013 (10μM) for 15 minutes prior to stimulation with and without EGF (50ng/ml) or S1P (5μM) for 10 minutes. (a) The western blot is of anti-HER2 immunoprecipitates probed with HRP-anti-phosphotyrosine antibody and demonstrates that S1P, but not EGF increases the tyrosine phosphorylation of HER2; (b) bar chart showing quantification of the effect of S1P, EGF and JTE013 on HER2 tyrosine phosphorylation for n=3 experiments.

Fig. 6 Role of HER2 in regulating S1P-induced activation of ERK-1/2 in MDA-MB-453 cells. MDA-MB-453 cells were pre-treated with scrambled siRNA or a HER2 siRNA (100 or 400nM, 48 hours) to knock down expression of HER2 or ErbB2 inhibitor II (at indicated concentrations) for 10 min prior to stimulation with and without S1P (at the indicated concentrations) or EGF (50ng/ml) or phyto-S1P (1 or 5μM) or FTY720 (5μM) for 10 minutes. (a) western blots showing the effect of siRNA (400nM) knock down of HER2 on S1P- (1μM and 5μM) and EGF-stimulated activation of ERK-1/2. In addition, cell lysates were immunoprecipitated with anti-HER2 antibody and analysed for ERK-1/2 activity with MBP as the substrate. The autoradiograph shows the siRNA knock down of HER2 reduces S1P- (5μM, 10 min) but not EGF-stimulated activation of ERK-1/2 (upper panel). Total ERK-2 inputs are also shown in the lower panel. The bar chart demonstrates quantification of the kinase assay using Cherenkov counting (p<0.01 for S1P-stimulated HER2 siRNA-treated cells versus S1P-stimulated scrambled siRNA-treated cells, n=4). Also shown is the siRNA knock down of HER2 (Mr=185kDa) detected on western blots probed with anti-HER2 antibody. The bar chart shows that siRNA knock down of HER2 had no effect on cell viability as assessed in the MTT assay; (b) bar chart showing quantification of the effect of siRNA knockdown of HER2 on S1P (1μM)- and EGF-stimulated ERK-1/2 activation; (c) western blot showing the effect of ErbB2 inhibitor II on S1P (5μM)-stimulated activation of ERK-1/2 and lack of effect on the response to EGF. Also shown; is a bar chart of the effect of ErbB2 inhibitor II on S1P- and EGF-stimulated ERK-1/2 activation; (d) western blot showing the effect of siRNA (100nM) knock down of HER2 on phyto-S1P- (1μM) stimulated activation of ERK-1/2. Also included is a bar chart showing quantification of the effect of HER2 siRNA on the phyto-S1P-induced activation of ERK-1/2 activation. These are representative results from three separate experiments. Results in (b-d) are expressed as P-ERK-1/2:ERK-2 ratios for n=3 experiments.
Phosphorylated ERK-1/2 was detected on western blots with anti-phosphorylated ERK-1/2 antibody. Blots were also probed with anti-ERK-1/2 antibody to ensure equal protein loading.

**Fig. 7** EGF-stimulated activation of ERK-1/2 occurs via a divergent signaling pathway compared with S1P in MDA-MB-453 cells. MDA-MB-453 cells were pre-treated with AG 1478 (0.1 or 1μM, 15 minutes) or siRNA EGF receptor (100nM, 48 hours) prior to stimulation with and without S1P (5μM) for 10 minutes. (a) and (c) Western blot showing the effect of (a) AG 1478 and (c) siRNA knock down of EGF receptor on the EGF- and S1P-stimulated activation of ERK-1/2. Also shown in (c) is the siRNA knock down of EGF receptor (Mr=170kDa) detected on western blots probed with anti-EGF receptor antibody. Bar charts showing quantification of the effect of (b) AG 1478 and (d) siRNA knock down of EGF receptor on EGF- and S1P-stimulated ERK-1/2 activation. Results in (b) and (d) are expressed as P-ERK-1/2/ERK-2 ratios for n=3 experiments. In (a) and (c), phosphorylated ERK-1/2 was detected on western blots probed with anti-phosphorylated ERK-1/2 antibody. Blots were also probed with anti-ERK-1/2 antibody to ensure equal protein loading.

**Fig. 8** The requirement for EGF receptor in the S1P stimulation of ERK-1/2 in MCF-7 cells. (a) Bar chart showing relative expression of S1P_{1-5} mRNA transcript in MCF-7 cells, determined using QRT-PCR. MCF-7 Neo cells (b) or MCF-7 HER2 cells (c) were pre-treated with either vehicle (DMSO), AG 1478 or AG 879 at the indicated concentrations for 15 minutes and then treated with and without S1P (1μM) for 5 minutes. The western blots show inhibition of S1P-stimulated ERK-1/2 by AG 1478 but not AG 879. In (b) and (c), phosphorylated ERK-1/2 was detected on western blots probed with anti-phosphorylated ERK-1/2 antibody. Blots were also probed with anti-ERK-2 antibody to ensure equal protein loading. Results in (b) and (c) are representative from three separate experiments.

**Fig. 9** Heregulin-stimulated activation of ERK-1/2 does not involve S1P_{4}. MDA-MB-453 cells were pre-treated with AG 1478 (100nM) or AG 879 (1μM) or CAY10444 (10μM) or JTE013 (5μM) for 15 minutes before treatment with heregulin (25ng/ml) for 10 minutes. The western blot shows the lack of effect of JTE013 and CAY10444 on the S1P-stimulated activation of ERK-1/2. Phosphorylated ERK-1/2 was detected on western blots probed with anti-phosphorylated ERK-1/2 antibody. Blots were also probed with anti-ERK-1/2 antibody to ensure equal protein loading. Results are representative of three separate experiments.

**Abbreviations**
ADAM, a disintegrin and metalloprotease; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERBB2, epidermal growth factor receptor 2 (or HER2); ERK, extracellular signal regulated kinase; GPCR, G protein-coupled receptor; HGF, hepatic growth factor; LPA, lysophosphatidic acid; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PTX, pertussis toxin; S1P, sphingosine 1-phosphate; S1P_{n}, S1P receptor, where n=1-5; SK, sphingosine kinase.
Fig. 1

(a) HER2 (185kDa)

231 453
MDA-MB

(b)

Relative mRNA abundance (fold over GAPDH ± SEM)

| Genes | S1P₁ | S1P₂ | S1P₃ | S1P₄ | S1P₅ |
|-------|------|------|------|------|------|
|       | 0.25 | 0.2  | 0.15 | 0.1  | 0.05 |
Fig. 2

(a)  

(b)
Fig. 3

(a)

S1P$_2$

S1P$_3$

S1P$_4$

% of Maximal S1P Response ± s.d.

Ligand [nM]

Ligand [nM]

Ligand [nM]
Fig. 3

(b) 

![Images showing Western blot analysis](image)

Control: S1P EGF

Vehicle: S1P EGF

JTE013: S1P EGF

CAY10444: S1P EGF

(c) 

P-ERK-1/ERK-2 ratio

![Graph showing ERK-1/2 ratio](image)

Control: S1P EGF

Vehicle: S1P EGF

JTE013: S1P EGF

CAY10444: S1P EGF

(d) 

P-ERK-1/ERK-2 ratio

![Graph showing ERK-1/2 ratio](image)

Control: S1P EGF

Vehicle: S1P EGF

JTE013: S1P EGF

CAY10444: S1P EGF

SEW

S1P

1 10 (µM)
Fig. 4

(a) Scrambled siRNA +       - +       -
S1P4 siRNA - +       - +

(b) P<0.05 P-ERK-1/2 / ERK-2 ratio

Scrambled siRNA
S1P4 siRNA

Downloaded from Jour-nal.org on July 9, 2020
Fig. 4

(c)

Rel. mRNA Abundance (% of scrambled siRNA effect + SEM)

- S1P2
- S1P3
- S1P4

Scrambled siRNA
S1P4 siRNA

ERK2

S1P4 siRNA
Scr siRNA

- + HEK-
+ - HA-S1P4

Vector
HEK-HA-S1P4

S1P4
Fig. 4 (d)

- No antibody
- Scrambled
- S1P₄ siRNA
- S1P₄ transfected

- Scrambled
- S1P₄ siRNA
- S1P₄ AB
Fig. 4
(d) continued

Scrambled siRNA

S1P2 AB

S1P2 siRNA
Fig. 4

(e)
Fig. 5

(a) IP: HER2
WB: P-Tyr

- S1P  EGF  - S1P  EGF

- JTE013

P-HER2 (185kDa)

(b) P<0.01  P<0.01

S1P  -  +  -  -  +  -
EGF  -  -  +  -  -  +

-  JTE013
Fig. 6

(a) ERK-2
P-ERK-1
P-ERK-2
-S1P (5µM)     EGF
- S1P (1µM)

HER2 siRNA
Scrambled siRNA

- + - + - + - +
+ - + - + - +

- EGF          S1P

ERK-2

HER2
P-ERK-1
P-ERK-2

Scrambled siRNA

- + - + - + - +
+ - + - + - +

- EGF          S1P

ERK-2

HER2 siRNA

Scrambled siRNA

- + - + - + - +
+ - + - + - +

- EGF          S1P

ERK-2

HER2 siRNA

Scrambled siRNA

- + - + - + - +
+ - + - + - +

- EGF          S1P

ERK-2

HER2 siRNA

Scrambled siRNA

- + - + - + - +
+ - + - + - +

- EGF          S1P

ERK-2
Fig. 6

(a) continued

![Graph showing OD$_{570}$ values for HER2 siRNA and Scrambled siRNA](graph.png)
Fig. 6

(b)

![Graph showing P-ERK-1/2 / ERK-2 ratio for Scrambled siRNA and HER2 siRNA with S1P and EGF treatments. The graph indicates a statistically significant difference (P<0.05) between the two siRNA treatments at the S1P and EGF conditions.](http://www.jbc.org/Downloaded from)
ErbB2 inhib II (μM)

C  S1P  EGF  C  S1P  EGF  C  S1P  EGF

0  25  50

P-ERK-1  P-ERK-2  ERK-2

P<0.05

P<0.01

P<0.05

P-ERK-1/2/ERK-2 ratio
Fig. 6 (d)

Scrambled siRNA  
HER2 siRNA  

| Phyto S1P | Scrambled siRNA | HER2 siRNA |
|-----------|-----------------|------------|
| -         | +               | +          |
|           | -               | +          |
|           | +               | -          |

| P-ERK-1   | P-ERK-2         |
|-----------|-----------------|
| HER2      | ERK-2           |

P<0.05

![Bar graph showing P-ERK-1/2 / ERK-2 ratio](http://www.jbc.org/Downloaded_from)
Fig. 7  

(a) 

AG 1478 (µM)      0.1       1         0.1        1         0.1       1  
S1P EGF

(b) 

P<0.05

AG1478 (100nM)           +        +        +        +        +        +  
AG1478 (1µM)               +        +        +        +        +        +  

P<0.05

P-ERK-1/2 / ERK-2 ratio
(c) 

**P-ERK-1/2 / ERK-2 ratio**

|               | P-ERK-1 | P-ERK-2 | ERK-2 |
|---------------|---------|---------|-------|
| Mock          |         |         |       |
| EGFR siRNA    |         |         |       |
| EGFR siRNA    |         |         |       |

- S1P       
- EGF

(d) 

**P-ERK-1/2 / ERK-2 ratio**

|       | P-ERK-1/2 / ERK-2 ratio |
|-------|-------------------------|
|       |                         |
|       |                         |
|       |                         |

*P<0.05*

- Mock
- EGFR siRNA
Fig. 8

(a)

Rel. mRNA Abundance (fold over actin ± SEM)

S1P$_1$ S1P$_2$ S1P$_3$ S1P$_4$ S1P$_5$
Fig. 8

(b) MCF-7 Neo

(c) MCF-7 HER2

AG 879 (μM) - - 10 1 - - - - 10 1 - -
AG 1478 (μM) - - - - 1 0.1 - - - - 1 0.1

S1P
Fig. 9

Heregulin by guest on July 9, 2020http://www.jbc.org/Downloaded from
Sphingosine 1-phosphate receptor 4 uses HER2 (ErbB2) to regulate extracellular signal regulated kinase-1/2 in MDA-MB-453 breast cancer cells

Jaclyn S. Long, Yuko Fujiwara, Joanne Edwards, Claire L. Tannahill, Gabor Tigyi, Susan Pyne and Nigel J. Pyne

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