Novel Mechanism of Lapatinib Resistance in HER2-Positive Breast Tumor Cells: Activation of AXL

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Abstract
HER2-directed therapies, such as trastuzumab and lapatinib, are important treatments for breast cancer. However, some tumors do not respond or develop resistance to these agents. We isolated and characterized multiple lapatinib-resistant, HER2-positive, estrogen receptor (ER)-positive breast cancer clones derived from lapatinib-sensitive BT474 cells by chronic exposure to lapatinib. We show overexpression of AXL as a novel mechanism of acquired resistance to HER2-targeted agents in these models. GSK1363089 (foretinib), a multitkine inhibitor of AXL, MET, and vascular endothelial growth factor receptor currently in phase II clinical trials, restores lapatinib and trastuzumab sensitivity in these resistant cells that exhibit increased AXL expression. Furthermore, small interfering RNA to AXL, estrogen deprivation, or fulvestrant, an ER antagonist, decreases AXL expression and restores sensitivity to lapatinib in these cells. Taken together, these data provide scientific evidence to assess the expression of AXL in HER2-positive, ER-positive patients who have progressed on either lapatinib or trastuzumab and to test the combination of HER2-targeted agents and GSK1363089 in the clinic. [Cancer Res 2009;69(17):6871–8]

Introduction
HER2 is one of the four members of the ErbB receptor tyrosine kinase protein family. Overexpression of HER2 in human breast tumors correlates with aggressive tumor growth and poor clinical prognosis. Thus, HER2 is an important therapeutic target in breast cancer, and several drugs that target HER2 are currently in clinical use. Lapatinib is a selective, potent, small-molecule, dual inhibitor of HER1 and HER2 that is approved in combination with capecitabine for the treatment of patients with advanced or metastatic breast cancer whose tumors overexpress HER2 and who have received prior therapy including an anthracycline, a taxane, and trastuzumab (1).

Although overexpression of HER2 correlates with sensitivity to growth inhibition by lapatinib, not all cells that overexpress HER2 respond to lapatinib (2), and resistance to lapatinib develops in some patients and/or tumors during chronic exposure to the drug. The factors that confer primary or acquired resistance to lapatinib are not well characterized. Several hypotheses including PTEN loss are reported to be associated with trastuzumab resistance in HER2-positive breast cancer (3, 4). However, lapatinib inhibits growth of trastuzumab-resistant breast cancer cells (5, 6) and shows clinical efficacy in patients who have received prior therapy with an anthracycline, a taxane, and trastuzumab (1). Moreover, low PTEN expression or PIK3CA mutations did not preclude response to lapatinib in primary tumors from women with HER2-overexpressing locally advanced breast cancer who received 6 weeks of lapatinib monotherapy before standard neoadjuvant treatment (7). Multiple mechanisms of resistance have been reported for tyrosine kinase inhibitors that selectively target HER1, such as gefitinib and erlotinib. For example, mutation of threonine 790 to methionine (T790M; ref. 8) and MET amplification (9) confer resistance to gefitinib and erlotinib. Other studies show that HER3 could remain phosphorylated and escape inhibition by HER1-targeted agents (10).

AXL is a membrane-bound receptor tyrosine kinase containing a kinase domain closely related to MET and an extracellular domain resembling that of neural cell adhesion molecules (11). Overexpression of AXL is associated with poor prognosis and increased invasiveness of human cancers and has been reported in breast (12, 13), colon (14), esophageal (15), thyroid (16), ovarian (17), gastric (18), renal (19), glioma (20), and lung (21) cancers. Overexpressing the cDNA for AXL yielded a tyrosine-phosphorylated 140 kDa protein with transforming ability (22, 23). The oncogenic potential of AXL lies within the tyrosine kinase domain, which can be activated by overexpression independent of ligand binding (24) or by binding to growth arrest–specific gene 6 (25). Recent studies report that increased expression of AXL may play a role in resistance to imatinib in gastrointestinal stromal tumors that express cKit (26) and in resistance to chemotherapy in AML (27) as well as in lung (28) and ovarian (29) cancers.

We established lapatinib-resistant clones from BT474 human breast cancer cells, which are estrogen receptor (ER)-positive, progesterone receptor (PgR)-positive, HER2-positive, and lapatinib-sensitive, and identified increased AXL expression as a novel mechanism of resistance to lapatinib and trastuzumab. AXL kinase inhibition by GSK1363089 (foretinib), a multitkine inhibitor targeting AXL, MET, and vascular endothelial growth factor receptor (VEGFR), restores lapatinib and trastuzumab sensitivity in these cells. ER deprivation or blockade restores lapatinib sensitivity by down-regulating AXL in these cells. These data implicate the development of novel therapeutic approaches for treating HER2-positive breast tumors.

Materials and Methods
Cell lines and reagents. Human breast carcinoma cell line BT474 was purchased from the American Type Culture Collection. Lapatinib, GSK1363089 (Supplementary Fig. S1), GSK690693, and NVP-BEZ-235 were
synthesized by GlaxoSmithKline. Trastuzumab was purchased from AmersourceBergen. Stock solutions of all drugs were prepared in DMSO, except trastuzumab, and stored at −20°C.

**Generation of lapatinib-resistant clones from BT474 cells in vitro.** BT474 cells were exposed to increasing concentrations of lapatinib from 0.5, 1, 2, 3, and 5 μmol/L. Single-cell cloning was done by serial dilution or isolation with cloning cylinders and expanded with 10% fetal bovine serum culture medium containing 0.5 to 1 μmol/L lapatinib. The R5 and J4 clones were selected from BT474 cells exposed to 1 and 3 μmol/L lapatinib, respectively. D3 and D25 were cloned from cells exposed to 5 μmol/L lapatinib. BT474 parental clones B1, B6, and B12 were selected by single-cell plating and maintained in RPMI 1640 containing 10% fetal bovine serum.

**Cell growth inhibition assay.** Cell growth inhibition was determined via CellTiter-Glo assay (Promega) according to the manufacturer's protocol and methylene blue cell viability method (2). Approximately 24 hours after plating, cells were exposed to compounds with 2- or 3-fold serial dilutions alone or the combination of two agents at a constant molar-to-molar ratio of 1:1 or as indicated. Cells were incubated with the compounds in culture medium containing 10% fetal bovine serum for 3 days. IC50 values were determined described previously (2).

** Colony formation assay.** Plates were plated at 10,000 to 50,000 per well in a 24-well plate in RPMI 1640 containing 10% fetal bovine serum. The following day, compounds, at the indicated dilutions, were added to the cells. For estrogen-deprived conditions, medium was removed and replaced with phenol red–free RPMI 1640 containing 10% charcoal-stripped serum with the indicated treatment. Cells were fixed and stained with 50% ethanol containing 0.1% methylene blue.

**Cell apoptosis assays: DNA fragmentation and caspase-3/7 activation.** Cell apoptosis was measured using Cell Death ELISAPlus kit (Roche) and Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions.

**Immunoprecipitation and Western blotting.** Protein extraction, immunoblotting, and immunoprecipitation were done as described previously (2). The antibodies used in immunoprecipitation are phosphotyrosine (Sigma), HER1 and HER2 (LabVision), HER3 (Santa Cruz Biotechnology), AXL (Cell Signaling), and p85 (Upstate). The primary antibodies used for immunoblotting are HER1 (LabVision), HER2 (Cell Signaling), HER3 (Santa Cruz Biotechnology), AXL, phospho-HER2(Y1221/1222), phospho-AKT(S473), AKT, BAD, phospho-BAD(S112), phospho-BAD(S136), phospho-FoxO1/3(α4/T24/T32), cyclin D1 (Cell Signaling), extracellular-signal-regulated kinase (ERK), phospho-ERK(T202/Y204; Santa Cruz Biotechnology), and β-actin (Abcam). The membranes were developed with Odyssey Infrared Imaging System (LI-COR Biosciences).

**Quantitative reverse transcription-PCR.** RNA was purified by Trizol extraction and purification with RNasey kit (Qiagen) following the manufacturer's instructions. cDNA was generated by High-Capacity Reverse Transcription kit (Applied Biosystems) using random primers. Gene expression was quantified with TaqMan universal master mix using TaqMan gene expression assays for AXL (HS00924357_m1), ESR (HS01046818_m1), and Pdgf (HS00172813_m1) on the ABI 7900 (Applied Biosystems).

**Data analysis.** All experiments were done as least two independent assays. Data are summarized as mean ± SD. Statistical analysis of the results was done by Student's t test for unpaired samples. P < 0.05 was considered statistically significant.

**Results**

**Selection and characterization of lapatinib-resistant, HER2-positive, ER-positive breast tumor cell lines.** BT474 breast cancer cells were chronically exposed to lapatinib (1, 3, or 5 μmol/L) in vitro and cells that developed resistance were selected. Twenty-one lapatinib-resistant cell clones were established from BT474 cells and characterized, four of which (J4, D3, D25, and R5) are shown in Fig. 1. Five control lapatinib-sensitive clones were also derived from BT474 cells cultured in the absence of lapatinib. Figure 1A shows representative growth curves of these cell lines in the presence of variable concentrations of lapatinib. The IC50 values of lapatinib-resistant cell lines were >3 μmol/L, ~50-fold higher than the IC50 of BT474 cells (0.071 μmol/L) or lapatinib-sensitive clones (B1, B6, B12. 0.051, 0.072, and 0.096 μmol/L, respectively). The lapatinib-resistant cell lines were also resistant to growth inhibition by HER-targeted tyrosine kinase inhibitors such as gefitinib, erlotinib, BI-2992 (Supplementary Table S1), and trastuzumab (Supplementary Table S1; Supplementary Fig. S2), but they remain sensitive to taxol or HS990 inhibitors (17-dimethylamoethyleno-17-demethoxy-geldanamycin and 17-allylamino-17-demethoxygeldanamycin; Supplementary Table S1). The lapatinib-resistant cell lines were more sensitive to NVP-BEZ-235, a phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin inhibitor, and GSK690693, an AKT inhibitor, than parental BT474 cells (Supplementary Table S1). Growth of the lapatinib-sensitive and lapatinib-resistant cells were not significantly inhibited by CI-1040, a MEK inhibitor, dasatinib, a Src/Abll dual inhibitor, and insulin-like growth factor-1 receptor inhibitors (cil3, BMS-695735; Supplementary Table S1).

The gene copy number and the mRNA and protein levels of HER1, HER2, and HER3 are similar in the lapatinib-resistant cell lines compared with the parental BT474 and clones (Fig. L8 and C; Supplementary Table S2). The HER2 kinase domain (exons 18-24), which was sequenced in genomic DNA from BT474 and BT474-J4 cells, has no mutations. We also confirmed that the DNA sequences of TP53 (homozygous mutation, 853G>A), PDKCA (heterozygous mutation, 333G>C), KRAS (wild-type), and PTEN (wild-type) are identical in BT474 and BT474-J4 cells (data not shown).

**Effect of lapatinib on phosphorylation of AKT and ERK in the lapatinib-resistant BT474 cells.** The phosphorylation status of HER family kinases and downstream targets of PI3K/AKT and ERK signaling was determined for BT474 and BT474-J4 cells (Fig. 1B and C; Supplementary Table S3). Lapatinib inhibited tyrosine phosphorylation of HER1, HER2, and HER3 in both lapatinib-sensitive BT474 cells and lapatinib-resistant BT474-J4 cells but decreased phosphorylation of AKT(S473), BAD(S136), FoxO1/3α(T24/T32), and ERK and reduced expression of cyclin D1 in only BT474 cells, suggesting that an alternative pathway to HER2 is responsible for activation of AKT in the resistant cells (Fig. 1B and C; Supplementary Table S3). Lapatinib decreased phospho-BAD(S112) to a greater extent in BT474 cells (76%) than in BT474-J4 cells (32%; Fig. 1C; Supplementary Table S3). HER4 expression was below the limit of detection by Western blot in both BT474 and BT474-J4 cells. Both BT474 and BT474-J4 cells were not sensitive to CI-1040 as determined by cell growth inhibition (Supplementary Table S1), although ERK phosphorylation was decreased by CI-1040 (Fig. 1C). NVP-BEZ-235 and GSK 690693 decreased the phosphorylation of AKT(S473) and BAD(S112) and the phosphorylation of BAD(S112), respectively, and both compounds reduced the expression of cyclin D1 in both BT474 and BT474-J4 cells (Fig. 1C; Supplementary Table S3). GSK690693 induced phosphorylation of AKT(S473) in BT474 cells, as reported previously (30), and in BT474-J4 cells (Fig. 1C). This observation is consistent with published reports of other AKT inhibitors and likely represents a previously hypothesized feedback pathway (30, 31). In addition, the effects of GSK690693 were analyzed on phospho-BAD(S136) and phospho-FoxO1/FoxO3α(T24/T32) that showed decreased phosphorylation ≥50% in both BT474 and BT474-J4 cells (Supplementary Table S3). Control experiments showed that the differences in the levels of...
Increased expression and activation of AXL in the lapatinib-resistant BT474 cells. Analysis by Western blot using a phosphotyrosine antibody detected a protein band at ~140 kDa of varying intensities in all the 17 lapatinib-resistant clones tested, which was not detected in BT474 cells and lapatinib-sensitive parental clones (Fig. 2A; data not shown). Mass spectrometry-based peptide sequencing identified this protein as AXL (Supplementary Fig. S4). This result was confirmed when the same band was detected only in the lapatinib-resistant BT474-J4 cells by Western blot analyses using an AXL-specific antibody (Fig. 2A).

Quantitative reverse transcription-PCR (qRT-PCR) showed that high levels of AXL mRNA were expressed in BT474-J4 (Ct = 20.7 ± 0.2) and additional lapatinib-resistant cell lines when compared with BT474 cells (Ct = 34.6 ± 1.0). Ct values for glyceraldehyde-3-phosphate dehydrogenase from BT474-J4 and BT474 cells are 23.6 ± 0.2 and 23.5 ± 0.2, respectively.

To further characterize the mechanism of increased AXL expression in lapatinib-resistant BT474-J4 cells, we determined the gene copy number and analyzed the promoter and coding regions of AXL. Quantitative PCR showed a slight (~2-fold) increase in AXL DNA copy number in the BT474-J4 lapatinib-resistant cell line when compared to the BT474 cells. Nucleotide changes were not detected by DNA sequencing within the promoter area of AXL using the genomic DNA and the coding region of AXL using cDNA from BT474 and BT474-J4 cells. We determined the DNA methylation status of the AXL promoter with bisulfite sequencing. Results indicated several methylation sites within the screened AXL promoter area for BT474 and lack of these sites in lapatinib-resistant BT474-J4 cells (Supplementary Fig. S5).

These results suggest that hypomethylation in the promoter region may play a role in the increased expression of AXL in BT474-J4 lapatinib-resistant cells and are consistent with recent reports that hypomethylation of the AXL promoter correlates with AXL overexpression (27, 32).

Inhibition or down-regulation of AXL restores lapatinib sensitivity. The role of AXL in acquired lapatinib resistance was
tested by treating BT474-J4 and the additional five lapatinib-resistant clones including D3, D25, and R5 with lapatinib in the presence and absence of GSK1363089, a potent small-molecule inhibitor of MET, VEGFR (33), and AXL (IC50 = 11 nmol/L). The mRNA expression levels of VEGFR were below detection by Affymetrix array (<50 MAS5 intensity) and the protein expression level of MET and absence of GSK1363089, a potent small-molecule inhibitor of MET, VEGFR (33), and AXL (IC50 = 11 nmol/L). The mRNA expression levels of VEGFR were below detection by Affymetrix array (<50 MAS5 intensity) and the protein expression level of MET and absence of GSK1363089, a potent small-molecule inhibitor of MET, VEGFR (33), and AXL (IC50 = 11 nmol/L). The mRNA expression levels of VEGFR were below detection by Affymetrix array (<50 MAS5 intensity) and the protein expression level of MET and absence of GSK1363089, a potent small-molecule inhibitor of MET, VEGFR (33), and AXL (IC50 = 11 nmol/L). 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Estrogen deprivation and fulvestrant down-regulates AXL expression and restores lapatinib sensitivity in the lapatinib-resistant BT474 cells. We determined the ER and PgR status in BT474 cells and six lapatinib-resistant cell lines and verified that BT474 cells are ER-positive and PgR-positive, whereas all the lapatinib-resistant clones are ER-positive and PgR-negative (Fig. 4A). In comparison with the parental BT474 cells, the resistant clones displayed a range of ER expression of 1.6- to 1.8-fold by Affymetrix analysis (Supplementary Fig. S8), 0.3- to 2.5-fold by qRT-PCR, and 0.1- to 2.8-fold by Western blot analysis. Therefore, the effect of estrogen deprivation (a surrogate model for aromatase inhibition, such as treatment with letrozole), fulvestrant, an ER antagonist, and tamoxifen, an antiestrogen, on lapatinib sensitivity of BT474-J4 cells was determined. BT474-J4 cells cultured in estrogen-depleted medium were sensitive to lapatinib (Fig. 4C and D). Fulvestrant also restored lapatinib sensitivity to BT474-J4 cells (Fig. 4B and C), whereas tamoxifen had a much weaker effect (data not shown). Intriguingly, both estrogen-depleted medium and fulvestrant down-regulated the expression of AXL mRNA (data not shown) and protein (Fig. 4D). These effects were reversed by addition of 17β-estradiol (Fig. 4C and D). AXL down-regulation by estrogen deprivation or fulvestrant is unexpected and novel, implicating that lapatinib in combination with ER blockers, such as fulvestrant, or aromatase inhibitors, such as letrozole, could be effective combinations in the clinic.

Genotypic and gene expression changes associated with acquired lapatinib resistance in HER2-positive, ER-positive BT474 cells. DNA copy number analysis performed on four lapatinib-sensitive cell lines and four lapatinib-resistant cell lines revealed similar HER2 amplification levels; however, several low-level copy number differences (~3-5 copies) were observed between lapatinib-sensitive and lapatinib-resistant cell lines.
Global gene expression profiling was done to compare differences between lapatinib-sensitive cells (BT474, B1, B6, and B12) and lapatinib-resistant cells (J4, D3, D25, and R5) using the Affymetrix HG-U133Plus2 chip. Approximately 250 genes were differentially expressed between sensitive and resistant clones, which were statistically significant with \( P < 0.001 \) and 
\[ \geq \text{2-fold} \] 
cutoff (data not shown). Highlights of these data, including 54 representative loci, are displayed graphically in Supplementary Fig. S8 using Ward’s method of hierarchical clustering. Genes that were differentially expressed between lapatinib-sensitive and lapatinib-resistant clones represent those involved in cell signaling pathways (e.g., AKT2, DUSP6, and PLK2), cell matrix interactions (e.g., CPE, ETV5, and LGALS3), hormonal regulation (e.g., PGR, GATA3, and STC2), and epigenetic modification (e.g., HDAC1 and SMYD2). These data show that the acquisition of lapatinib resistance in BT474 cells from increased expression of AXL is associated with significant alterations at the mRNA level.

Gene expression profiles for BT474-J4 lapatinib-resistant cell lines treated with lapatinib, GSK1363089, and the combination in comparison with BT474 cells treated with lapatinib alone are illustrated in Supplementary Fig. S8B. In contrast to either single-agent treatment, BT474-J4 cells treated with lapatinib in combination with GSK1363089 for 24 h strongly down-regulated genes associated with cell signaling (e.g., DUSP6, DUSP4, PLK2, ICMT, and RET), cell matrix interactions (e.g., ETV4, ETV5, and FAM46C), cell growth and survival (e.g., CCND1, CDC25C, and BNI1), and metabolism (e.g., CTPS, FABP5, and LRBP8) and up-regulated genes associated with apoptosis and cell cycle arrest (e.g., TNFSF10 and TP53INP1), a profile very similar to parental BT474 cells treated with lapatinib alone (Supplementary Fig. S8B). These results show that GSK1363089 restores the ability of lapatinib to modulate gene expression in BT474-J4 cells.

Discussion

The goal of this study was to identify mechanisms by which HER2-positive, ER-positive human breast cancer cells develop resistance to lapatinib. For this purpose, lapatinib-resistant clones were selected from BT474 breast cancer cells in vitro, and 21 independent lapatinib-resistant cell lines were established and characterized. These cell lines were also resistant to other HER-targeted agents. Our data provide strong evidence that increased expression of AXL confers a mechanism of acquired resistance to lapatinib in this model system. This mechanism of lapatinib resistance has not been identified previously, although lapatinib-acquired resistant cell lines have been reported by Xia and colleagues (34).

AXL is a membrane-bound receptor tyrosine kinase, which has been reported to play a role in resistance to imatinib as well as to chemotherapy (26–28). This current study provides definitive evidence that AXL can play a direct role in acquired lapatinib resistance in HER2-positive, ER-positive breast cancer cells as follows: (a) BT474 lapatinib-resistant cell lines have increased AXL expression, whereas BT474 lapatinib-sensitive cell lines do not; (b) lapatinib sensitivity is restored in BT474 lapatinib-resistant cells by GSK1363089, a potent inhibitor of AXL, MET, and VEGFR, whereas compounds inhibiting MET or the VEGFR family do not have this effect; and (c) AXL-targeted siRNA restores lapatinib sensitivity in BT474-J4 cells, which are also sensitive to HER2-targeted siRNA in combination with GSK1363089. These findings provide important information to support the clinical development of combination drug therapies for treating HER2-positive breast cancer and in the proposed crosstalk among ER, AXL, and HER family receptors (Fig. 5).
Phosphorylation of AXL is reported to be associated with HER2 signaling (35). It has also been shown that both AXL and HER3 directly bind p85 for downstream AKT activation (36, 37). Our data show that neither lapatinib nor GSK1363089 alone interrupts the interaction between AXL and p85 in BT474-J4 cells, but lapatinib in combination with GSK1363089 does (Fig. 2A). In contrast, lapatinib inhibits phosphorylation of HER2 and HER3 and interrupts the interaction between HER3 and p85 in both BT474 and BT474-J4 cells. This indicates that suppression of both AXL and HER3 phosphorylation is necessary to block the interaction of AXL with p85, leading to the disruption of the PI3K/AKT pathway.

All of the lapatinib-resistant BT474 clones tested express ER but have lost PgR expression. ER-positive, PgR-negative tumors with high expression of HER1 or HER2 are more aggressive and are associated with resistance to single hormonal therapy, such as tamoxifen in the clinic (38–40). This report shows a novel mechanism by which HER2-positive, ER-positive breast cancer cells acquire resistance to lapatinib by increased expression of AXL. Previous studies suggest that AXL expression correlates with expression of ER in breast cancer cells (13). We show that estrogen deprivation (as a surrogate for aromatase inhibitor treatment such as letrozole) or the ER antagonist fulvestrant reduced AXL expression and inhibited cell growth in combination with lapatinib.

Estrogen supplementation in the culture medium restored the overexpression of AXL and lapatinib resistance in BT474-J4 cells (Fig. 4C and D). These data suggest that lapatinib in combination with ER blockers such as letrozole or fulvestrant could potentially treat and/or delay the onset of lapatinib resistance in HER2-positive and hormone-positive breast cancer. To test this hypothesis, the ideal scenario would be to measure the expression of AXL in pretreatment and post-treatment biopsies of patients treated with lapatinib who have progressed; however, this is very difficult to do as access to these samples is limited. The beneficial effect of lapatinib in combination with estrogen depletion has been observed in the recently reported EGF30008 clinical trial showing that lapatinib significantly improved the efficacy of an aromatase inhibitor letrozole in patients with known HER2-positive, hormone receptor–positive metastatic breast cancer when compared with letrozole alone (41). Although this study was first-line treatment of letrozole ± lapatinib, ~50% of patients on the study had received prior endocrine therapy in the adjuvant setting. Of the patients who received prior endocrine therapy, the overwhelming majority had tamoxifen.

Furthermore, GSK1363089, a multikinase inhibitor that targets AXL, restores lapatinib sensitivity in HER2-positive breast cancer cells that overexpress AXL. GSK1363089 is currently in phase II clinical trials for gastric, head and neck, and papillary renal cancer.

Figure 5. Model for ER/AXL-dependent lapatinib resistance and the crosstalk among ER, AXL, and HER family receptors in ER-positive and HER2-positive breast cancer cells. Coactivation of AXL with HER2 leads to downstream PI3K/AKT and ERK signaling which are essential to cell growth and survival. ER blockers such as estrogen deprivation or fulvestrant reduce the expression of AXL. Inhibiting both HER2 and AXL is necessary to reduce the downstream PI3K/AKT and ERK signaling and cell growth.

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