Gefitinib-mediated Reactive Oxygen Specie (ROS) Instigates Mitochondrial Dysfunction and Drug Resistance in Lung Cancer Cells*

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Background: Drug resistance contributes to treatment failure and cancer-related mortality.

Results: Increased ROS due to chronic gefitinib treatment was blocked by mito-Tempo, and restored mitochondrial function.

Conclusion: Gefitinib-mediated ROS instigates mitochondrial dysfunction in resistant lung cancer cells.

Significance: Antioxidants may alleviate ROS-mediated resistance in lung cancer cells.

Therapeutic benefits offered by tyrosine kinase inhibitors (TKIs), such as gefitinib (Iressa) and erlotinib (Tarceva), are limited due to the development of resistance, which contributes to treatment failure and cancer-related mortality. The aim of this study was to elucidate mechanistic insight into cellular perturbations that accompany acquired gefitinib resistance in lung cancer cells. Several lung adenocarcinoma (LAD) cell lines were screened to characterize epidermal growth factor receptor (EGFR) expression and mutation profile. To circumvent intrinsic variations between cell lines with respect to response to drug treatments, we generated gefitinib-resistant H1650 clone by long-term, chronic culture under gefitinib selection of parental cell line. Isogenic cells were analyzed by microarray, Western blot, flow cytometry, and confocal and transmission electron microscope. We observed that although chronic gefitinib treatment provided effective action against its primary target (aberrant EGFR activity), secondary effects resulted in increased cellular reactive oxygen species (ROS). Gefitinib-mediated ROS correlated with epithelial-mesenchymal transition, as well as striking perturbation of mitochondrial morphology and function. However, gefitinib treatment in the presence of ROS scavenger provided a partial rescue of mitochondrial aberrations. Furthermore, withdrawal of gefitinib from previously resistant clones correlated with normalized expression of epithelial-mesenchymal transition genes. These findings demonstrate that chronic gefitinib treatment promotes ROS and mitochondrial dysfunction in lung cancer cells. Antioxidants may alleviate ROS-mediated resistance.

Targeted agents against EGFR-driven lung tumors, including gefitinib (Iressa) and erlotinib (Tarceva), have proven beneficial among a subset of patients (1–4). These reversible tyrosine kinase inhibitors (TKIs) compete for ATP-binding pockets of intracellular receptor tyrosine residues, blocking receptor activation, recruitment of adaptor molecules, and aberrant signaling cascades. However, drug resistance to existing TKIs remains a major challenge and contributes to cancer-related mortality. Like several other anticancer therapies, the introduction of irreversible TKIs ultimately succumbs to resistance after initial responsiveness by cancer cells. Therefore, a better understanding of the processes and mechanisms that promote resistance is critical and imperative to finding interventions that will enable effective treatments.

Although certain genetic mutations strongly influence clinical response to TKIs (5–11), the dysregulation of cellular processes (such as metabolic alterations) may contribute to drug resistance. Furthermore, studies that employ different cell lines to investigate response to anticancer agents present difficulty with interpretation due to intrinsic variations that exist within such cancer cell lines. However, a widely agreed characteristic of drug-resistant cancer cells is the differential expression of EMT genes, which is normally required during early embryogenesis (12–15). For example, the loss of classic epithelial markers, such as E-cadherin, during EMT correlates with the expression of mesenchymal markers, such as vimentin. Epithelial functions can significantly impact membrane integrity, and hence the attenuation of genes that are critical to membrane functions may play a leading role in tumor development or progression. Consistent with EMT, the formation of motile cells from parental cells that lack such motility have been shown to be a hallmark of invasive and metastatic phenotypes displayed by tumors (12, 16, 17). The expression of EMT genes has been further associated with poor prognosis (13–15). Hence, drug resistance in this study was defined by the expression profile of selected, well characterized EMT genes.

Although intrinsic cellular processes result in reactive oxygen specie (ROS) production, cells typically possess robust homeostatic mechanisms for maintaining redox balance.

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2The abbreviations used are: EGFR, epidermal growth factor receptor; KRAS, Kirsten rat sarcoma viral oncogene homolog; ROS, reactive oxygen species; EMT, epithelial-mesenchymal transition; TKI, tyrosine kinase inhibitor; PDnse, pyruvate dehydrogenase; ANOVA, analysis of variance; CLN, clone; mTempo, mito-Tempo; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase.
Gefitinib-mediated ROS Contributes to Drug-resistant LADs

Unsurprisingly, external factors, such as radiation or chemotherapy, can impact the coordinated regulation of redox reactions and hence disrupt cellular ROS balance. In this study, we found that chronic gefitinib treatments resulted in increased ROS levels, which correlated with EMT. We wanted to elucidate how the increase in cellular ROS and EMT impacted cellular processes and functions. The findings from this study provide novel mechanistic insight into cellular perturbations that accompany acquired gefitinib resistance and identify a strategy that could alleviate ROS-mediated resistance in lung cancer cells.

EXPERIMENTAL PROCEDURES

Cell Lines—MRC-9 (normal “non-cancer”), H1650, H1734 and H1975 lung adenocarcinoma cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA) in 2011. All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in an open air incubator with full medium consisting of RPMI 1640 medium (Sigma) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

Generation of Gefitinib-resistant H1650 Clone—Parental H1650 cells were subjected to chronic gefitinib exposure for 10–12 weeks. Cells were continuously cultured and maintained under gefitinib selection. For applications, gefitinib-resistant H1650G clones were seeded in 6- or 96-well plates and cultured under the same conditions (no drug treatment and 2% serum overnight) as the parental sensitive H1650 cells prior to subsequent assay procedures.

Microarray and EGFR Mutation Analysis—Gene expression profiling of gefitinib-sensitive (H1650) and -resistant (H1650G) cells was performed by Phalanx Biotech Group (Belmont, CA). EGFR mutation profile (exons 18–21), as well as KRAS selection (exon 2), was carried out using the QClamp mutation test kit from DiaCarta (Hayward, CA).

Reagents and Inhibitors—The tyrosine kinase inhibitors, gefitinib (Iressa) and erlotinib (Tarceva), were purchased from LC Laboratories (Woburn, MA). EGFR and a cell viability assay kit were purchased from Promega (Madison, WI). A cell death detection (TUNEL) kit was purchased from Roche Applied Science and used according to prescribed instructions. Mito-TEMPO was purchased from Santa Cruz Biotechnology (Dallas, TX).

ROS Measurements—ROS measurements were undertaken using CellRox oxidative stress reagents from Molecular Probes by Life Technologies and the total ROS assay kit from Affymetrix eBioscience (San Diego, CA) according to manufacturer's instructions. ROS detection was measured by Tecxan (infinite M1000) and flow cytometry (BD Biosciences FACSCalibur 4C), respectively.

Cell Cycle Analysis—Cell cycle analysis between parental versus resistant H1650 isogenic cells was undertaken as follows. ~1 × 10⁶ cells were pipetted and washed into a flow tube. Cells were centrifuged, blotted, and resuspended in 250 µl of hypotonic stain and hyperton stop buffers. Cells were incubated at 4 °C for >1 h before data acquisition by flow cytometry.

Mitochondria Isolation—The isolation of mitochondria fraction from cultured cells was undertaken according to protocol from the kit that was purchased from Abcam (San Francisco, CA). Pyruvate dehydrogenase antibody cocktail was purchased from MitoSciences (Eugene, OR).

Measurement of Extracellular Acidification Rate and Oxygen Consumption Rate—XF glycolysis and metabolic stress kits from Seahorse Bioscience were employed in the measurement of oxygen consumption rate and extracellular acidification rate according to the manufacturer’s instructions. XF96 flux analyzer (Seahorse Bioscience) was used for measurements and data acquisition.

Quantitative Real-time PCR—Custom-designed PCR plates with various metabolic, mitochondrial, and EMT targets were purchased from Bio-Rad Laboratories and employed for real-time PCR. cDNA from gefitinib-sensitive or -resistant H1650 cells were prepared using SABioscience RT² First Strand kit (Valencia, CA). A Bio-Rad CFX96 C1000 thermal cyclyer was used for data acquisition and analysis.

Western Blots and Antibodies—Samples were typically run in 8 or 12% gels and transferred onto nitrocellulose from Bio-Rad. Membranes were probed with specific antibodies according to the manufacturer’s suggestions after blocking in 5% milk/TBST (Tris-buffered saline and Tween 20). Primary Tyr(P)-20, β-actin, catalase, and GAPDH anti-mouse monoclonal antibodies were from Santa Cruz Biotechnology. All other antibodies, including anti-rabbit phospho- and total polyclonal antibodies against mutant (L858R) and wild-type EGFR, AMPK, ACC, AKT, ERK1/2, vimentin, CD44, fascin, keratin-19, E-cadherin, and cleaved caspase3 were from Cell Signaling (Beverly, MA).

Microscopy—Fixed cells (4% paraformaldehyde) were permeabilized, blocked, and stained with specific antibodies (1:100) overnight. Alexa Fluor 488 anti-rabbit secondary antibody from Invitrogen was used (1:400) for indirect immunofluorescence. Coverslips were mounted on slides using SureMount® with DAPI. Imaging was carried out using 710 Zeiss confocal microscope with a 63× oil immersion objective.

Statistical Analysis—Statistical analysis was done using Student’s t test, one-way ANOVA, or two-way ANOVA. p values of <0.05 were considered significant. Significance for each figure is noted as follows: #, p < 0.05, *, p < 0.01, **, p < 0.001, and ***, p < 0.0001.

RESULTS

Characterization of EGFR-TKI Lung Cancer Cell Lines—The MRC-9, a normal (non-cancer) lung cell line, along with H1650, H1734, and H1975 lung adenocarcinoma (LAD) cell lines, was subjected to biochemical analysis to characterize EGFR expression and mutation profile. MRC-9 and H1734 cells possess wild-type EGFR gene and H1650 cells possess exon 19 deletions (DelE746-A750), whereas H1975 cells possess a point mutation in exon 21 (L858R) and secondary mutation (T790M) attributed to drug resistance (Fig. 1A). Responses to TKIs have been detailed to be effective within a subset of tumors that possess certain EGFR mutations (5–11). At different concentrations (0.5 or 1.0 µM), gefitinib demonstrated stronger efficacy in comparison with erlotinib, as measured by decreased phosphorylation of tyrosine kinase residues (Fig. 1, B and C). Under the same conditions, H1975 cell line with secondary drug-resistant mutation failed to respond to TKI treatments in comparison
CD44 is a type I transmembrane glycoprotein linked with cell-signaling, and tumor microenvironment remodeling (18–22), strongly associated with increased cell motility, migration, and drug resistance (13–15). Our microarray data confirmed differential expression of several EMT genes associated with drug resistance. In agreement with previous studies that have relied on previously described EMT markers to determine drug resistance, we observed increased invasive and migratory potentials within H1650G clones relative to parental cells (data not shown). Conversely, keratin-19 (KRT19), an intermediate filament protein mainly expressed in active flat, elongated cell morphology was evident in gefitinib-resistant H1650G cells as compared with the round-shaped parental cells (data not shown).

As would be expected, attenuation of EGFR phosphorylation correlated with the inhibition of canonical downstream targets (namely, ERK1/2 and AKT), as well as increased cell death in H1650G-resistant clones (Fig. 2, A and B). EGFR (exons 18–21) and KRAS (exon 2) mutation analyses failed to detect any new mutations in both parental resistant and drug-resistant H1650 cells (data not shown). As expected, attenuation of EGFR phosphorylation correlated with the inhibition of canonical downstream targets (namely, ERK1/2 and AKT), as well as increased cell death in H1650G-resistant clones (Fig. 2, A and B). EGFR (exons 18–21) and KRAS (exon 2) mutation analyses failed to detect any new mutations in both parental resistant and drug-resistant H1650 cells (data not shown). As expected, attenuation of EGFR phosphorylation correlated with the inhibition of canonical downstream targets (namely, ERK1/2 and AKT), as well as increased cell death in H1650G-resistant clones (Fig. 2, A and B). EGFR (exons 18–21) and KRAS (exon 2) mutation analyses failed to detect any new mutations in both parental resistant and drug-resistant H1650 cells (data not shown).

FIGURE 1. Validation of EGFR expression and mutation profile in lung cell lines. A, under basal conditions, selected lung cell lines were screened. MRC-9 (normal, non-cancer) and H1734 lung cancer cell lines possess wild-type EGFR. In exon 21, EGFR point mutation (L858R) was confirmed in H1975 cells. Gefitinib or erlotinib at low (0.5 μM) or high (1.0 μM) dosages was applied for 3 h prior to exogenous EGFR stimulation (50 ng/ml) for 15 min. t-EGFR, total EGFR. B, stronger response to gefitinib was demonstrated by H1650 cells at both concentrations, as measured by the inhibition of canonical tyrosine 1068 residue (Tyr(P)-1068 (pY1068)) and global tyrosine residue (Tyr(P)-20 (pY20)). The H1650 cell line possesses in-frame exon 19 deletions (DelE746-A750) and is sensitive to TKIs. C, in addition to the L858R point mutation, the H1975 cell line possesses a secondary EGFR mutation (T790M) ascribed to TKI resistance and demonstrated a poor response to both inhibitors at high concentrations.

HER2 has been linked with increased cell growth of ovarian carcinoma (23–25). Fascin (FSCN1) has been implicated in the development and regulation of lamellipodia, filopodia, and cell migration (28–30). Increased FSCN1 expression has been detailed to correlate with enhanced invasion and motility of tumors, including, breast, colon, and prostate cancer (31–33). In agreement with these studies, we observed increased invasive and migratory potentials within H1650G clones relative to parental cells (data not shown). Conversely, keratin-19 (KRT19), an intermediate filament protein mainly expressed in epithelial cells (34), was strongly down-regulated in gefitinib-resistant H1650G cells relative to the parental controls (Fig. 3B). Both microscopic and biochemical analyses confirmed enhanced expression of canonical EMT marker, vimentin in H1975 cell lines, as well as the H1650G gefitinib-resistant clone in comparison with parental H1650 cells (Fig. 3, C–F). The H1975 cell line possesses a secondary EGFR point mutation (T790M) ascribed to drug resistance. Furthermore, a distinctive flat, elongated cell morphology was evident in gefitinib-resistant clones as compared with the round-shaped parental cells (data not shown).

**Gefitinib-mediated ROS Instigates Mitochondrial Dysfunction**—Differential EMT gene expression and distinct cell morphology between parental resistant versus drug-resistant H1650 isogenic cells prompted further studies on the perturbation of cellular processes. We measured the levels of dihydroethidium as a function of oxidative stress in parental and resistant cells. Although increasing ROS was observed over the measured time intervals across these cells, ROS levels in H1650G clone (CLN) were significantly accentuated in comparison with H1650 wild-type cells (WT; p < 0.001) (Fig. 4A). The data suggest that chronic gefitinib treatment promotes cellular ROS levels. Flow cytometry was further used to confirm increased ROS levels in gefitinib-resistant clones, which were similar to H2O2-treated controls (Fig. 4B). Biochemical data demonstrated that parental cells expressed catalase (antioxidant enzyme) under basal or H2O2 treatment, but this was not detected in the drug-resistant clone (Fig. 4C). As would be expected, vimentin expression was evident only within gefitinib-resistant cells and was up-regulated in response to hydrogen peroxide (Fig. 4C). Due to the potential effects of unregulated ROS on cellular processes, we employed transmission electron microscopy to analyze the...
organelles of parental cells versus gefitinib-resistant clones. Surprisingly, a striking difference in both mitochondrial size and number was evident between the two cell groups (Fig. 4, D and E). As demonstrated by multiple images, larger and relatively fewer mitochondria were present in the resistant clones as compared with parental cells (Fig. 4, D and E). We next inves-
tigated mitochondrial respiratory and metabolic functions. Oxygen consumption rate was severely diminished in gefitinib-resistant clones as compared with parental cells following treatment with trifluoromethoxy carbonylcyanide phenylhydrazone (Fig. 4F). Trifluoromethoxy carbonylcyanide phenylhydrazone is a potent uncoupler of oxidative phosphorylation in mitochondria; it disrupts the synthesis of ATP by transporting protons across cell membranes. Decreased ATP levels were also confirmed in the gefitinib-resistant clones (data not shown). Cytosol glycolytic process, measured as glucose-induced extracellular acidification rate, did not show any difference between the isogenic cells (Fig. 4G). Taken together, we demonstrate that gefitinib-mediated ROS impacts mitochondrial morphology and respiratory function.

**Gefitinib Resistance Correlates with Attenuated Pyruvate Dehydrogenase Expression**—Given the observed effects of chronic gefitinib treatment on the mitochondria, the expression of several mitochondria-associated genes was assessed. At the message level, no differences were observed across selected mitochondrial targets between parental resistant or gefitinib-resistant cells (data not shown). Furthermore, biochemical analyses failed to show any differences, except for pyruvate dehydrogenase (PDnse) expression, which was attenuated in multiple gefitinib-resistant clones (Cln-1 and Cln-2) as compared with the WT parental cells (Fig. 5A). Decreased PDnse expression was further confirmed by microscopy (Fig. 5B). Pyruvate dehydrogenase is an important metabolic enzyme that is required for the conversion of pyruvate, the end product of glycolysis to acetyl-CoA for maximum energy (ATP) generation via the citric acid cycle in the mitochondria. Further analyses of glycolytic genes upon treatment with 2-deoxyglucose confirmed specific attenuation of PDnse expression in drug-resistant cells (Fig. 5C). 2-Deoxyglucose is a glucose analogue that cannot be metabolized for ATP generation via the glycolytic pathway. Following the isolation of mitochondrial fraction from isogenic cells, we confirmed decreased PDnse expression in resistant clones (Fig. 5D). We verified that the inhibition of PDnse expression was not directly linked to the EGF receptor. Knockdown (siRNA) of EGFR or overexpression of EGFR plasmids in parental H1650 cells provided no effect on PDnse expression (data not shown). Pyruvate dehydrogenase is a multi-enzyme complex, consisting of E1 (pyruvate dehydrogenase), E2 (dihydrolipoamide acetyltransferase), E3 (dihydrolipoamide dehydrogenase), and E3bp (E3-binding protein) subunits. We confirmed significant inhibition of E3bp and E1α/ E1β subunits in the mitochondrial fraction of drug-resistant clone as compared with parental cells (Fig. 5, E and F). Up-regulated ROS levels in gefitinib-resistant (Cln-1) or H2O2-treated
cells correlated with attenuated expression of PDnse (Fig. 5G), which is in agreement with the reported effects of oxidative stress as a target of pyruvate dehydrogenase complex (35).

**ROS Scavenger Partially Rescues Gefitinib-resistant Phenotypes**—To demonstrate a direct link between gefitinib-mediated ROS and mitochondrial dysfunction, a fresh set of H1650 cells was cultured in the presence of gefitinib, mito-Tempo (a mitochondria-specific ROS scavenger), or combined gefitinib/mito-Tempo treatments over several weeks. Untreated cells were taken as parental wild-type controls. Cells from the different treatment groups were analyzed by Western blot, microscopy, and flow cytometry. At the protein level, expression of mesenchymal marker (vimentin), which was highly up-regulated in the gefitinib-treated group, was decreased upon gefitinib/mito-Tempo (mTempo) treatments (Fig. 6A). Likewise, attenuated expression of canonical epithelial marker (E-cadherin) in gefitinib-treated cells was rescued in the presence of gefitinib/mTempo treatments (Fig. 6A). Further analysis by microscopy demonstrated similar results of the EMT gene profile upon the combination of mTempo with gefitinib treatments (Fig. 6B). However, there was no significant -fold change of several EMT genes upon gefitinib/mTempo treatments at the message level (data not shown). Flow cytometry demonstrated normalized ROS levels in gefitinib/mTempo treatments as compared with gefitinib-only cells (Fig. 6C). Subsequent assessment of the mitochondria revealed a rescue of
mitochondria morphology and oxygen consumption rate in gefitinib/mTempo-treated cells (Fig. 6, D and E). Finally, due to the fact that increased ROS was instigated by chronic gefitinib treatments, the drug was withdrawn from previously resistant cells. Gefitinib withdrawal over a 12-week period correlated with the loss of vimentin and FSCN1 expression, whereas the expression of E-cadherin, KRT19, and PDnse was restored (Fig. 6F). Taken together, these data demonstrate that gefitinib-mediated ROS promotes EMT and mitochondrial dysfunction, which correlate with resistance of lung cancer cells (Fig. 7).

**DISCUSSION**

This study details perturbation to cellular processes and functions that accompany acquired gefitinib resistance of lung cancer cells. We observed that although chronic gefitinib treatments provided effective action against its primary target (aberrant EGFR activity), it also promoted increased cellular ROS levels. Up-regulated ROS correlated with EMT, a classic hallmark of drug-resistant tumors (13–15, 36). EMT genes, such as E-cadherin and vimentin, possess cytoskeletal, membrane, and/or cell adhesion functions; hence perturbations to such targets may impact tumor metastasis and drug failure (resistance). Dramatic changes to mitochondria morphology and decreased respiratory function were associated with gefitinib resistance. Specific attenuation of pyruvate dehydrogenase expression, decreased oxidative phosphorylation, and ATP levels are in agreement with mitochondrial dysfunction. However, gefitinib treatments in combination with a mitochondria-specific ROS scavenger, mTempo, were sufficient to partially reverse EMT and restore mitochondria functions (Fig. 6).

These observations require further investigation and cautious interpretation as a previous study has shown that antioxidants can accelerate lung cancer progression in mice (37). However, others have reported beneficial anticancer effects by antioxidants (38–40). Our study was mainly limited to ROS from chronic gefitinib treatments, as well as the use of a mitochondrial...
dria-specific ROS scavenger (mTempo). Different antioxidants, namely N-acetylcysteine and vitamin E, that were used in the reported study (37), as well as different experimental conditions, may account for the divergent findings.

Cancer treatments utilizing radiation or chemotherapy have been strongly associated with increased ROS levels, a mechanism of action that is partly responsible for apoptosis of cancer cells (41–44). As would be expected, oxidative stress may have several effects on cellular processes and gene regulation. For example, pyruvate dehydrogenase complex has been described as a target of oxidative stress in ischemic brain injury (35). Paradoxically, increased ROS and apoptosis of gefitinib-resistant cells were matched with abnormal cell cycle and increased proliferation in our study. Our data suggest that functional loss or mutations of antioxidant genes (such as catalase) may contribute to unregulated and increased cellular ROS. Robust expression of catalase under basal or hydrogen peroxide treatment was demonstrated by parental sensitive cells but undetected in the drug-resistant clone (Fig. 4C). Conversely, vimentin expression within gefitinib-resistant cells was up-regulated in response to hydrogen peroxide (Fig. 4C), supporting the regulation of EMT by ROS.

The mitochondrion is a major organelle within a cell, and it possesses the ability of autonomous replication independent of the nucleus. Hence, the mitochondrion is aptly described as the “powerhouse” of a cell given its critical roles in several processes, including cellular respiration and metabolism. It is therefore unsurprising that drug treatments with deleterious effects on the mitochondria will almost certainly impact cellular processes. In this study, gefitinib-driven ROS correlated with a specific dysfunction of the mitochondria. However, a partial rescue of mitochondrial function upon treatments with...
a mitochondria-specific ROS scavenger (mTempo) may suggest the presence of ROS from additional sources. Beyond modulation of EMT gene expression, how does ROS link with drug-resistant cancer cells, and with the mitochondria specifically? It would appear that alterations to mitochondrial morphology and functions represent cellular adaptations by drug-resistant cancer cells. As has become an emerging pattern of tumors, cellular alterations that would otherwise prove fatal to normal cells have instead presented adaptations that enable tumors to thrive. For example, in this study, decreased oxidative phosphorylation and mitochondrial ATP production within gefitinib-resistant clones failed to impact cell survival and proliferation. Mitochondrial dysfunction mediated by ROS may therefore represent a response to toxic tumor microenvironment from drug treatments. However, further studies are required to fully understand the sources of ROS, as well as how it targets the mitochondria. Perhaps ROS causes alteration to well-coordinated processes, such as mitochondrial fission or fusion.

Together, these findings suggest that antioxidants could potentially provide therapeutic benefits by attenuating TKI-induced ROS and EMT. The identification of phenotypes and markers associated with drug resistance may provide useful diagnostic or therapeutic applications.

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