E-Cadherin/ROS1 Inhibitor Synthetic Lethality in Breast Cancer

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**ABSTRACT**

The cell adhesion glycoprotein E-cadherin (CDH1) is commonly inactivated in breast tumors. Precision medicine approaches that exploit this characteristic are not available. Using perturbation screens in breast tumor cells with CRISPR/Cas9-engineered CDH1 mutations, we identified synthetic lethality between E-cadherin deficiency and inhibition of the tyrosine kinase ROS1. Data from large-scale genetic screens in molecularly diverse breast tumor cell lines established that the E-cadherin/ROS1 synthetic lethality was not only robust in the face of considerable molecular heterogeneity but was also elicited with clinical ROS1 inhibitors, including foretinib and crizotinib. ROS1 inhibitors induced mitotic abnormalities and multinucleation in E-cadherin-defective cells, phenotypes associated with a defect in cytokinesis and aberrant p120 catenin phosphorylation and localization. In vivo, ROS1 inhibitors produced profound antitumor effects in multiple models of E-cadherin-defective breast cancer. These data therefore provide the preclinical rationale for assessing ROS1 inhibitors, such as the licensed drug crizotinib, in appropriately stratified patients.

**SIGNIFICANCE:** E-cadherin defects are common in breast cancer but are currently not targeted with a precision medicine approach. Our preclinical data indicate that licensed ROS1 inhibitors, including crizotinib, should be repurposed to target E-cadherin–defective breast cancers, thus providing the rationale for the assessment of these agents in molecularly stratified phase II clinical trials. Cancer Discov; 8(4); 498–515. ©2018 AACR.

**INTRODUCTION**

E-cadherin defects are frequently found in breast cancer (>13%) and gastric cancer (>14%) and are particularly prevalent in lobular breast cancers, which account for 15% of all mammary carcinomas (1). CDH1 encodes a calcium-dependent plasma membrane–bound cell–cell adhesion glycoprotein (2). In epithelial cells, E-cadherin forms homotypic adhesive complexes, known as adherens junctions (AJ) that control cell–cell contact, the contractility of cells, and ultimately the integrity of epithelial cell layers (3). Although the extracellular domain of E-cadherin interacts with E-cadherin molecules on adjacent cells, the intracellular domain interacts with, and controls, a number of proteins including p120 catenin (p120), α-catenin, γ-catenin, β-catenin, receptor tyrosine kinases, and a series of plasma membrane–associated receptors and cytoskeletal proteins (2). Loss of E-cadherin function causes a wide variety of phenotypes ranging from defects in cell migration and the orientation of the mitotic spindle, as well as dysregulation of cell–cell adhesion and anoikis resistance (reviewed in ref. 3).

In lobular breast cancer, loss of E-cadherin expression occurs early on in the tumorigenic process and is seen in up to 90% of cases, often co-occurring with mutations in the PI3K coding gene PIK3CA (4). Lobular breast cancers tend to be estrogen receptor (ER)– and progesterone receptor (PR)–positive and ERBB2 amplification–negative and to have a low Ki67 index and a luminal A–intrinsic subtype (1, 5–7). Although these biomarkers might predict a favorable response to endocrine therapy, retrospective analyses of two recent clinical trials (BIG 1-98 and ABCSG-8) suggest that a subset of patients with invasive lobular breast cancer (ILC) have poorer responses to endocrine therapy when compared with those with invasive ductal carcinomas (IDC) that display similar biomarkers (8, 9). Furthermore, pathologic complete response rates after neoadjuvant chemotherapy are low in ILC (10, 11), suggesting that additional approaches are required to target this disease. In other breast cancer subtypes, E-cadherin expression might also influence patient outcome. For example, in triple-negative breast cancer, the prognosis of patients with E-cadherin–negative tumors is significantly worse than those with E-cadherin–positive disease (12, 13).

At present, it is not clear whether actionable or pharmacologically tractable E-cadherin synthetic lethal effects can be identified that are likely to work clinically. Such clinically actionable synthetic lethal effects might be expected to be relatively resilient to additional molecular changes and to
operate in the face of the high degree of molecular diversity that exists in cancer (i.e., hard synthetic lethal effects; ref. 14). In the data presented below, we illustrate that the combined use of multiple, distinct, in vitro, ex vivo, and in vivo model systems and the exploitation of different functional profiling modalities (genetic and chemical screens) can be used to identify robust and actionable E-cadherin synthetic lethal interactions. The most notable synthetic lethality we identified in this way was between E-cadherin and the ROS1 receptor tyrosine kinase, an effect that is clinically actionable using ROS1 inhibitors such as crizotinib or foretinib.

RESULTS

Integrated Genetic and Small-Molecule Screens Identify a ROS1/E-Cadherin Synthetic Lethal Effect

To identify candidate therapeutic targets for breast cancers with loss of E-cadherin, we used CRISPR/Cas9 mutagenesis in MCF7 breast tumor cells (ERα-positive, luminal A, PIK3CA mutant; described hereafter as MCF7parental cells) to generate daughter clones (MCF7^ROS1, MCF7^ROS1, and MCF7^ROS1) with frame-shift mutations in CDH1 and loss of E-cadherin expression (Fig. 1A and B; Supplementary Fig. S1). Compared with MCF7^ROS1 cells, E-cadherin–defective cells displayed a rounded morphology also seen in breast tumor cells harboring naturally occurring E-cadherin mutations (Fig. 1C). We used MCF7^ROS1 and MCF7^ROS1 cells in two parallel functional screens to identify E-cadherin synthetic lethal effects: (i) a drug-sensitivity screen where we assessed the relative sensitivity of cells to an in-house curated library of 80 small-molecule inhibitors that are either in clinical use for the treatment of cancer or in late-stage clinical development (Fig. 1D; Supplementary Tables S1 and S2); and (ii) a parallel siRNA sensitivity screen, using siRNA SMARTpools (four different siRNAs targeting a single gene in each well) targeting >1000 cell-cycle control genes, kinase-coding genes or DNA repair–related genes (see Methods; Fig. 1E; Supplementary Table S3). The drug-sensitivity screens identified a series of candidate E-cadherin synthetic lethal drugs, including PF-03758309 (a PAK inhibitor), PF-03814735 (an Aurora kinase inhibitor), PL3K/mTOR inhibitors (BEZ-235, PF-04691502, and everolimus), and the ROS1/MET/ALK inhibitors (15) crizotinib (PF02341066, Pfizer) and foretinib (GSK1363089, GSK; Supplementary Table S4). Amongst the most significant hits in the siRNA screen was ROS1 (ROS proto-oncogene 1, receptor tyrosine kinase; Fig. 1E; Supplementary Table S3). We selected ROS1 for further study as (i) the synthetic lethality observed with ROS1 siRNA was statistically significant and (ii) the parallel small-molecule inhibitor screen identified ROS1 inhibitors (foretinib and crizotinib) as candidate synthetic lethal drugs, suggesting that the ROS1 effect might be clinically tractable.

The siRNA screen did not identify other targets of crizotinib or foretinib (e.g., MET, ALK, KDR, and AXL) as being candidate E-cadherin synthetic lethal effects (Supplementary Fig. S2). We also noted that in both MCF7 and MCF7^ROS1 isogenic systems, loss of E-cadherin expression caused upregulation of ROS1 protein but did not elicit changes in either MET or ALK expression (Fig. 1F and G; Supplementary Fig. S3), suggesting that enhanced ROS1 expression could represent a homeostatic response to E-cadherin loss; reexpression of E-cadherin in E-cadherin–defective MCF7^ROS1 cells reversed this ROS1 induction (Fig. 1H), suggesting causality.

In validation experiments, we found that each of the four individual ROS1 siRNAs from the ROS1 SMARTpool caused silencing of ROS1 and preferentially inhibited the E-cadherin–deficient MCF7^ROS1 clone and also an E-cadherin–deficient

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**Figure 1.** ROS1 inhibition is synthetic lethal with E-cadherin defects in isogenic models. A, Wild-type E-cadherin protein is lost in the CDH1 CRISPR/ Cas9 mutated MCF7^ROS1 clone. Western blot illustrating E-cadherin expression in parental MCF7 cells (MCF7parental) and the MCF7^ROS1 clone is shown. Upper band is a glycosylated isoform of E-cadherin, the lower band represents the nonglycosylated form. B, Confocal microscopy images of MCF7parental and MCF7^ROS1 cells, illustrating loss of E-cadherin expression (green) in MCF7^ROS1 cells. Nuclei are imaged with DAPI (blue). C, Light microscopy images of MCF7parental and MCF7^ROS1 cells, illustrating reduction in cell-cell contact in MCF7^ROS1 cells. D, Volcano plot illustrating AUC ratios (MCF7^ROS1/MCF7^ROS1) wild-type parental cells) of 80 small-molecule inhibitors assessed in the high-throughput screen. AUC ratio <1 indicates candidate E-cadherin synthetic lethal effects. Blue dots represent ROS1 inhibitors. E, Volcano plot of data from the siRNA SMARTpool sensitivity screens in cell lines described in B. Blue dot highlights ROS1 siRNA identified in the screen as selectively targeting E-cadherin–defective cells. F, ROS1 expression is upregulated in E-cadherin–defective cells. G, Western blot illustrating expression of ROS1, MET, and ALK in MCF10A CDH1−/− and MCF10A CDH1−/− cells. ACTIN expression is used as loading control. H, Ectopic expression of E-cadherin in MCF7^ROS1 E-cadherin–defective cells reduces ROS1 expression. Western blot illustrating ROS1 expression in E-cadherin–defective MCF7^ROS1 cells transfected with FLAG epitope-CDH1 cDNA. ACTIN is used as loading control.
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A

MCF7 PARENTAL
MCF7 A02
105 kDa
48 kDa
E-cadherin
ACTIN

B

DAPI
E-cadherin
MCF7 parental

MCF7 A02

C

MCF7 parental

MCF7 A02

D

ROS1 inhibitors

AUC (MCF7A02/MCF7Parental) Ratio

P value

E

Target E-cadherin-
defective

Target E-cadherin-
wild-type

F

MCF10A CDH1+/
MCF10A CDH1−/

ROS1
135 kDa
245 kDa

MET
135 kDa

ALK
245 kDa

E-cadherin
38 kDa

ACTIN

G

MCF10A CDH1+/
MCF10A CDH1−/

Blue = DAPI

H

MCF7 A02

FLAG–E-cadherin

245 kDa

ROS1

135 kDa

38 kDa

ACTIN
Figure 2. Validation of ROS1 synthetic lethality in E-cadherin-defective isogenic models. A, Western blot illustrating ROS1 silencing caused by four different ROS1 siRNAs (1, 2, 3, and 4) compared with two different nontargeting siRNAs (siCONT1 and siCONT2). Uncropped Western blot images are shown in Supplementary Fig. S20. B and C, Bar charts illustrating cell inhibition caused by ROS1 siRNAs in MCF7+/+/+ and MCF7−/− cells (B) and MCF10A CDH1+/+ and MCF10A CDH1−/− cells (C). NPI, normalized percentage inhibition [compared with siCONT (NPI = 1) and siPLK1 (NPI = 0)]. Error bars, SEM from three independent experiments. D, Bar chart illustrating the effect of 1 μmol/L foretinib or crizotinib in MCF7+/+/+ compared with two additional E-cadherin-defective clones, MCF7−/− and MCF7−/−. Error bars, SEM from three independent experiments. E, Western blot illustrating E-cadherin expression in MCF7+/+ cell line model transfected with a FLAG epitope-CDH1 cDNA expression construct. F, Surviving fraction data from MCF7+/+/+ and MCF7−/− and MCF7−/+/FLAG-CDH1 cell lines exposed to 1 μmol/L foretinib, crizotinib, and TAE684. Cells were transfected with FLAG-epitope-CDH1 cDNA and clones selected in G418. Clones expressing FLAG-E-cadherin were exposed to 1 μmol/L foretinib, crizotinib, or TAE684 for 6 continuous days, at which point cell viability was assessed. *P < 0.05, Student t test as shown. G, Dot chart illustrating cell survival effects of ROS1/MET/ALK inhibitors in MCF7+/+/+ and MCF7−/− cells. S, surviving fraction 50 (concentration required to cause 50% reduction in survival). H, and I, Dose-response survival curves in MCF10A CDH1+/+ and MCF10A CDH1−/− cells exposed to foretinib for 6 days (H) or 2 weeks (I). Error bars, SEM from three independent experiments. I, ANOVA P value MCF10A CDH1+/+ versus MCF10A CDH1−/− cells < 0.0001. Dose response in SLC34A2–ROS1 translocation–positive HCC78 cells shown is a positive control. J, Dose-response survival curves in MCF10A CDH1+/+ and MCF10A CDH1−/− cells exposed to the ROS1 kinase inhibitor PF-06463922 for 6 days. Error bars, SEM from three independent experiments. K, Dose-response survival curves in MCF7+/+ cells transfected with different concentrations of ROS1 siRNA SMART pool and subsequently exposed to foretinib for 6 days. Increasing concentration of ROS1 siRNA caused a dose-dependent reduction in foretinib SF50. Error bars, SEM from three independent experiments. L, Expression of a crizotinib-refractory p.G2032R mutant ROS1 fusion cDNA (18) causes crizotinib resistance in E-cadherin-defective MCF10A CDH1−/− cells. Cells were transfected with either a p.G2032R CD74–ROS1 CDH1 cDNA expression vector or a cDNA expression vector without a CD74–ROS1 insert (empty). Twenty-four hours after transfection, cells were exposed to crizotinib for a subsequent 6 days, at which point cell viability was assessed. Data show the median effects of three independent experiments. Error bars illustrate the SEM. ***, P < 0.001. Student t test as shown.
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**Figure A**
- Western blot analysis of ROS1 proteins in MCF7 parental, MCF7B04, and MCF7B05 cells.
- Proteins detected at 245 kDa and 48 kDa.

**Figure B**
- Graph showing the effect of ROS1 siRNA on cell survival.
- Three bars with **P < 0.05** and one with **P < 0.01**.

**Figure C**
- Graph showing the effect of ROS1 siRNA on cell survival.
- Three bars with **P < 0.05** and one with **P < 0.01**.

**Figure D**
- Graph showing the effect of Foretinib and Crizotinib on cell survival.
- Three bars with **P < 0.05**.

**Figure E**
- Western blot analysis of ACTIN and FLAG-E-cadherin proteins.
- Probes detected at 135 kDa and 48 kDa.

**Figure F**
- Bar graph showing the effect of 1 µmol/L concentration of various drugs on cell survival.
- Four bars with **P < 0.05**.

**Figure G**
- Graph showing the effect of various drugs on cell survival.
- Four bars with **P < 0.05**.

**Figure H**
- Graph showing the effect of 6-day drug exposure on cell survival.
- Three lines with **P < 0.05**.

**Figure I**
- Graph showing the effect of 2-week drug exposure on cell survival.
- Three lines with **P < 0.05**.

**Figure J**
- Graph showing the effect of PF-06463922 (mol/L) on cell survival.
- Three lines with **P < 0.05**.

**Figure K**
- Graph showing the effect of siROS1_1–5 nmol/L on cell survival.
- Seven lines with **P < 0.05**.

**Figure L**
- Graph showing the effect of various siROSI concentrations on cell survival.
- Seven lines with **P < 0.001** and one with **ns = not significant**.
Using this approach, we found E-cadherin protein deficiency to have a far greater correlation with the mutational status of the 23 recurrently mutated cancer driver proteins in the breast tumor cell line panel plus the E-cadherin mutational status of the breast tumor cell lines as the measure of target inhibition and the analysis using the \( \text{ROS1} > \text{inhibition} \); REVEALER ICs \( \leq 0.1 \) are regarded as negative correlation/features associated with sensitivity to target inhibition (Fig. 3F), supporting the hypothesis that E-cadherin status is an important determinant of sensitivity to ROS1 inhibition in breast cancer. We also assessed the sensitivity of the breast tumor cell line panel to foretinib or crizotinib and found that the E-cadherin–defective models were more sensitive to both inhibitors (Fig. 3G and H; Supplementary Table S8). The median sensitivity to either foretinib or crizotinib in the breast tumor cell line panel appeared to be independent of epithelial or mesenchymal status. For example, MCF10A cells do not undergo EMT upon losing E-cadherin expression (23) but did exhibit both foretinib and crizotinib sensitivity (Fig. 2H–J). Similarly, SUM149, a mesenchymal breast tumor cell line that has wild-type E-cadherin expression (24), was not sensitive to ROS1 inhibition, whereas E-cadherin–defective, epithelial cells (e.g., SUM44 and SKBR3) were (Supplementary Fig. S8). Furthermore, we did not find that expression

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**Figure 3.** Synthetic lethality of ROS1 inhibition in E-cadherin–deficient breast tumor cell lines. **A,** Western blot illustrating E-cadherin expression in 37 breast tumor cell lines (including MCF7 as positive control). Cell line names are color-coded according to the presence of CDH1 gene mutations, gene deletion events, and CDH1 promoter hypermethylation events. Uncropped Western blot images are shown in Supplementary Fig. S20. **B,** Schematic illustrating breast tumor cell line cohort sizes used to interrogate siRNA sensitivity screens (19). **C,** Bar chart illustrating E-cadherin expression of a subset of the breast tumor cell lines shown in A, determined by mass spectrometry, described in ref. 20. Western blot classification of E-cadherin status from these effects as nonlinear information correlation coefficients (IC), between 1 (perfect correlation/features associated with resistance to target inhibition) and -1 (perfect negative correlation/features associated with sensitivity to target inhibition); REVEALER ICs ≤0.1 or >0.1 are regarded as profound correlations (22). We carried out a REVEALER analysis using the ROS1 siRNA Z score data in the breast tumor cell lines as the measure of target inhibition and the mutational status of the 23 recurrently mutated cancer driver genes in the breast tumor cell line panel plus the E-cadherin protein classification described above as molecular features. Using this approach, we found E-cadherin protein deficiency to have a far greater correlation with ROS1 siRNA Z score (IC -0.5) than any of the other molecular features analyzed

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Mutations or other defects in key cancer driver genes or proteins and target inhibition data (in this case sensitivity to ROS1 siRNA) from tumor cell line profiling experiments to identify multiple, often complementary, molecular features that correlate with target inhibition, quantifying these effects as nonlinear information correlation coefficients (IC), between 1 (perfect correlation/features associated with resistance to target inhibition) and -1 (perfect negative correlation/features associated with sensitivity to target inhibition); REVEALER ICs ≤0.1 or >0.1 are regarded as profound correlations (22). We carried out a REVEALER analysis using the ROS1 siRNA Z score data in the breast tumor cell lines as the measure of target inhibition and the mutational status of the 23 recurrently mutated cancer driver genes in the breast tumor cell line panel plus the E-cadherin protein classification described above as molecular features. Using this approach, we found E-cadherin protein deficiency to have a far greater correlation with ROS1 siRNA Z score (IC -0.5) than any of the other molecular features analyzed (Fig. 3F), supporting the hypothesis that E-cadherin status is an important determinant of sensitivity to ROS1 inhibition in breast cancer. We also assessed the sensitivity of the breast tumor cell line panel to foretinib or crizotinib and found that the E-cadherin–defective models were more sensitive to both inhibitors (Fig. 3G and H; Supplementary Table S8). The median sensitivity to either foretinib or crizotinib in the E-cadherin–defective cohort was also similar to that in the SLC34A2–ROS1-translocated HCC78 tumor cell line model (Fig. 3G and H). The responses to ROS1 inhibitors observed in the tumor cell line panel appeared to be independent of epithelial or mesenchymal status. For example, MCF10A cells do not undergo EMT upon losing E-cadherin expression (23) but did exhibit both foretinib and crizotinib sensitivity (Fig. 2H–J). Similarly, SUM149, a mesenchymal breast tumor cell line that has wild-type E-cadherin expression (24), was not sensitive to ROS1 inhibition, whereas E-cadherin–defective, epithelial cells (e.g., SUM44 and SKBR3) were (Supplementary Fig. S8). Furthermore, we did not find that expression
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We did note an increase in ROS1 variants, although rare in our tumor cell line panel, did not appear to correlate with crizotinib/foretinib sensitivity.

**Figure 3. (Continued)** F, REVEALER analysis identifies E-cadherin status as an important determinant of ROS1 siRNA sensitivity. Heat map illustrates the REVEALER output identifying complementary genomic alterations associated with ROS1 siRNA sensitivity in the 34 breast tumor cell line panel described in B. IC scores and nominal P values with respect to the target are shown on the right side of the heat map. E-cadherin status demonstrated the most profound IC score −0.5 from 23 molecular features examined. G and H, Box whisker plots illustrating foretinib (G) or crizotinib (H) sensitivity in 12 breast tumor cell lines, defined by log2 AUC values. Individual AUC values are listed in Supplementary Table S8. Log2 AUC values for SLC34A2–ROS1 translocation-positive HCC78 cells are shown as a positive control. **, P = 0.003, Student t test; *, P value = 0.035, Student t test. WT, wild-type. I, Volcano plot of drug sensitivity effects in ex vivo cultured breast cancer explants (27) annotated according to CDH1 gene copy-number loss. Red dot highlights crizotinib as selectively targeting explants with CDH1 copy-number loss versus models with no CDH1 copy-number loss. Median AUC in explants with CDH1 copy-number loss = 0.0713, median AUC in explants without CDH1 copy-number loss = 0.138, P = 0.00127. Y-axis shows adjusted P value (−log10) from a t test comparing AUC in ex vivo explant models with CDH1 loss versus models with no CDH1 copy-number change. X-axis shows the negative value of the t statistic describing the difference in AUC means for ex vivo explant models with CDH1 loss versus models with no CDH1 copy-number change. Negative values suggest drug sensitivity in ex vivo explant models with CDH1 loss versus models with no CDH1 copy-number change.

of MET, ALK, pAKT, AKT, pERK, or ERK correlated with E-cadherin status nor drug sensitivity (Supplementary Fig. S9), suggesting that these were unlikely to be determinants of the synthetic lethal effects we identified. As ROS1 fusion genes are well-established biomarkers of ROS1 inhibitor sensitivity, we also analyzed paired-end RNA-sequencing data using ChimeraScan in order to identify ROS1 fusion genes in our tumor cell line panel (25, 26). Although ChimeraScan identified the SLC34A2–ROS1 fusion event in HCC78 lung tumor cells (the positive control), ROS1 fusions were not identified in any of the breast tumor cell lines (Supplementary Table S9). In addition, ROS1 copy number or ROS1 variants, although rare in our tumor cell line panel, did not appear to correlate with crizotinib/foretinib sensitivity (Supplementary Table S9). We did note an increase in ROS1 expression and pROS1 levels in E-cadherin–defective cells, similar to those seen in E-cadherin isogenic systems (Fig. 1F–H; Supplementary Fig. S10), suggesting that enhanced ROS1 expression could represent a homeostatic response to E-cadherin loss. Finally, we interrogated recently published data describing drug-sensitivity effects in ex vivo–cultured breast cancer explants (27) to identify drug-sensitivity effects associated with loss of E-cadherin. When querying these data for drug-sensitivity effects associated with CDH1 gene copy-number loss, we found sensitivity to crizotinib (PF-02341066; ref. 27) to be the most significant effect (Fig. 3I, P value 0.00127). The mean AUC for crizotinib was 0.0713 in explants with CDH1 copy-number loss (n = 5) and 0.138 in explants without CDH1 copy-number loss (n = 12; FDR 0.214).
ROS1 Inhibitors Elicit Synthetic Lethality in Endocrine-Resistant, E-Cadherin–Defective, Breast Tumor Cells and in Models of E-Cadherin–Defective Gastric Cancer

Given the central role of endocrine therapy in the treatment of ER+ breast cancers and the frequency at which endocrine resistance develops, especially in the advanced disease setting (28), we assessed whether CDH1 siRNA caused foretinib or crizotinib sensitivity in previously validated, endocrine therapy–resistant, MCF7 LTED cells (29). We found that CDH1 siRNA caused both foretinib and crizotinib sensitivity in MCF7 LTED cells (P < 0.0001, ANOVA, Fig. 4A–E) as it did in endocrine-sensitive MCF7 cells (Fig. 2G), suggesting that the E-cadherin foretinib/crizotinib synthetic lethal effects were somewhat independent of endocrine therapy sensitivity. We also found that ER+, de novo endocrine-resistant and E-cadherin–defective MDAMB134VI tumor cells, which were derived from a case of invasive lobular carcinoma (30), were sensitive to ROS1 inhibitors in both in vitro (Supplementary Fig. S11A) and in vivo experiments (Supplementary Fig. S11B–S11D), suggesting that ROS1 inhibition could also target E-cadherin–defective breast tumor cells in this particular setting.

We next assessed whether crizotinib or foretinib sensitivity extended to models of E-cadherin–defective diffuse gastric cancer, given the elevated frequency of E-cadherin defects in this cancer subtype. We found that either crizotinib or foretinib selectively targeted E-cadherin–defective gastric tumor cell lines (Supplementary Fig. S12A and S12B). The effects of crizotinib in additional gastric tumor cell lines have also been recently assessed (27, 31). We reanalyzed
Figure 5. Cell-cycle and mitotic defects in E-cadherin–defective cells. A, FACS plots illustrating increase in DNA content in E-cadherin–defective MCF7A02 cells exposed to foretinib. B, Enlarged image from A indicating >4N fraction. The fraction of cells with >4N DNA content is shown. C, Box whisker plots indicating fraction of cells with abnormal mitoses (e.g., multinuclear defects) in cells exposed to foretinib. ***, P = 0.0001; **, P = 0.0054, Student t test. Nuclear defects were visualized by confocal microscopy and a minimum of 200 cells were counted for each cell line. Data are representative of two independent experiments in each case. D, Illustrative confocal microscopy images indicating multinuclear phenotype in E-cadherin–defective MCF7A02 cells exposed to foretinib. E, Illustrative confocal microscopy images indicating multinuclear phenotype in E-cadherin–defective SKBR3 and BT549 cells and E-cadherin wild-type T47D and SUM149 cells exposed to 1 μmol/L foretinib. F, Box whisker plots indicating fraction of cells with abnormal mitoses (e.g., multinuclear defects) in cells exposed to foretinib. *, P = 0.0218, Student t test. Data are representative of two independent experiments in each case. G, Western blot illustrating increased p21 levels in the E-cadherin–defective MCF7A02 cells exposed to 1 μmol/L foretinib versus MCF7parental cells. (continued on next page)
these data and found that those tumor cell lines with reduced E-cadherin expression (defined by CDH1 mRNA levels) were more sensitive to crizotinib than those with higher E-cadherin expression (Supplementary Fig. S12C).

**ROS1 Inhibition Exacerbates p120 Catenin, Cleavage Furrow and Cytokinesis Defects in E-Cadherin–Defective Cells**

In investigating the cellular phenotypes associated with these E-cadherin–selective effects, we found that exposure of E-cadherin–defective cells to foretinib caused an increase in the proportion of cells with 4N DNA content (Fig. 5A and B); an increase in the frequency of cells with abnormal mitoses, particularly cells with multiple nuclei (Fig. 5C–P); an increase in expression of the G2-M DNA damage biomarker p21 (Fig. 5G); and an increase in cellular apoptosis as assessed by PARP cleavage and caspase 3/7 activity (Fig. 5H–L; Supplementary Fig. S13). These phenotypes were reminiscent of those seen in rhabdomyosarcoma tumor cells exposed to crizotinib (32) or chronic myeloid leukemia (CML) tumor cell lines, where foretinib elicits phenotypes associated with mitotic catastrophe (MC), including multinucleated giant cells, increased DNA content, and apoptotic cell death (33).

To assess what might cause such multinuclear phenotypes, we visualized cells exposed to crizotinib or foretinib using live cell microscopy. We found that in both E-cadherin wild-type and E-cadherin–defective cells, either foretinib or crizotinib extended the time cells spent in mitosis (Supplementary Fig. S14A and S14B). This extended mitosis had dichotomous effects, depending upon the status of E-cadherin. In E-cadherin wild-type cells, exposure to a ROS1 inhibitor resulted in the formation of two mononuclear daughter cells (Fig. 6A). In contrast, when exposed to a ROS1 inhibitor,
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**A**

- **MCF7** parental vehicle
  - 0: 14 35 49 56 70 (min)
- **MCF7** parental foretinib
  - 0: 21 56 76 84 98 (min)
- **MCF7** parental crizotinib
  - 0: 14 35 63 70 105 (min)

**B**

- **MCF10A**
  - Vehicle
  - p-p120 (Tyr228)
  - p120
  - E-cadherin
  - ACTIN

**C**

- **MCF7A02**
  - WCL
  - FLAG IP
  - GFP–empty
  - GFP–p120
  - FLAG–ROS1

**D**

- si**CONT** si**p120**
- 135 kDa
- 75 kDa
- p120
- ACTIN

**E**

- Fraction of cells with abnormal mitosis
- **si**p120**: **si**p120**
- **si**ROS1

**F**

- NPI
  - Student test
  - **si**p120**: **si**p120**
  - **si**ROS1

**G**

- **MCF7** parental
  - siControl
  - si**p120**
  - si**ROS1**
  - si**PLK1**
- **MCF7A02**
  - siControl
  - si**p120**
  - si**ROS1**
  - si**PLK1**
E-cadherin–defective cells initiated cytokinesis but failed to complete invagination of the cell membrane at the cleavage furrow, resulting in the formation of multinuclear cells (Fig. 6A). We noted that cytokinesis in E-cadherin–defective cells exposed to foretinib or crizotinib was characterized by prolonged membrane oscillation, starting at the onset of anaphase, cleavage furrow regression resulting in multinucleated cells, and also the formation of cells with lagging chromosomes (Fig. 6A; Supplementary Fig. S15; Supplementary Videos S1–S5).

Cytokinesis failure and a multinuclear phenotype have previously been associated with defective p120 catenin activity (p120; refs. 34, 35). Actomyosin contractility at the ingestion and cleavage furrow during anaphase and telophase is controlled by p120, which mediates these effects via binding to the central spindle protein MKLP1 and the GTPase RHOA (35). p120 catenin also normally interacts with E-cadherin at the cell membrane where it is tyrosine phosphorylated (36–38), raising the possibility that loss of E-cadherin could impair p120 function. We found that E-cadherin–defective tumor cells exhibited a reduction in p120 catenin levels (Fig. 6B), consistent with recent findings from a large-scale analysis of breast tumors with/without E-cadherin defects (39). Furthermore, as well as ROS1 immunoprecipitating with p120 (Fig. 6C), we found that ROS1 inhibition (i) exacerbated the p120 reduction seen in E-cadherin–defective cells; (ii) reduced tyrosine phosphorylation of p120; and (iii) reduced p120 levels at the cleavage furrow (Fig. 6B and C; Supplementary Fig. S16A and S16B). We also found that siRNA-mediated gene silencing of p120 (or ROS1) caused a multinuclear phenotype and elicited synthetic lethality in E-cadherin–defective but not E-cadherin wild-type cells (Fig. 6D–G). In normal epithelial cells, two opposing forces facilitate cytokinesis: (i) force provided by E-cadherin–mediated AJ at the apical membrane and (ii) force provided by the contraction of the actomyosin ring at the cleavage furrow (40). One model to explain our observations might be that loss of one of these opposing forces in E-cadherin–defective cells causes a greater reliance on processes and proteins that control cleavage furrow formation, such as p120 catenin. Our data suggest that ROS1 inhibitors exacerbate an existing p120 defect in E-cadherin–defective cells and impair cytokinesis to such an extent that abnormal mitoses could impair p120 function. We found that E-cadherin–defective tumor cells exhibited a reduction in p120 catenin levels (Fig. 6B), consistent with recent findings from a large-scale analysis of breast tumors with/without E-cadherin defects (39). Furthermore, as well as ROS1 immunoprecipitating with p120 (Fig. 6C), we found that ROS1 inhibition (i) exacerbated the p120 reduction seen in E-cadherin–defective cells; (ii) reduced tyrosine phosphorylation of p120; and (iii) reduced p120 levels at the cleavage furrow (Fig. 6B and C; Supplementary Fig. S16A and S16B). We also found that siRNA-mediated gene silencing of p120 (or ROS1) caused a multinuclear phenotype and elicited synthetic lethality in E-cadherin–defective but not E-cadherin wild-type cells (Fig. 6D–G). In normal epithelial cells, two opposing forces facilitate cytokinesis: (i) force provided by E-cadherin–dependent AJ at the apical membrane and (ii) force provided by the contraction of the actomyosin ring at the cleavage furrow (40). One model to explain our observations might be that loss of one of these opposing forces in E-cadherin–defective cells causes a greater reliance on processes and proteins that control cleavage furrow formation, such as p120 catenin. Our data suggest that ROS1 inhibitors exacerbate an existing p120 defect in E-cadherin–defective cells and impair cytokinesis to such an extent that abnormal mitoses form; this elicits a DNA-damage response (e.g., induction of p21 and γH2AX) and ultimately apoptosis. ROS1 inhibition has lesser effects when buffered by the activity of wild-type E-cadherin.

Consistent with the concept that E-cadherin defects might cause a dependency upon processes controlled by actomyosin networks, such as cleavage furrow maturation, we also noted that the RHO GTPase effector kinase CDC42BPA (CDC42-binding kinase, MRCKα), which controls actomyosin function (41), was also identified as a robust synthetic lethal effect in our earlier siRNA screens (Supplementary Fig. S4A and S4B). We validated this synthetic lethal effect in subsequent experiments, including those using a toolbox small-molecule MRCKα inhibitor (ref. 42; Supplementary Fig. S17A–S17D), suggesting that additional targets associated with these processes might exist in E-cadherin–defective cancers.

**DISCUSSION**

E-cadherin defects are a common characteristic of human tumors. Here, we show that ROS1 inhibition constitutes an E-cadherin synthetic lethal interaction that can be elicited with clinical inhibitors such as crizotinib or foretinib. These effects appear to be robust in the face of considerable molecular heterogeneity that exists among different isoform models, distinct tumor cell lines, ex vivo breast tumor explants, and different mouse models of E-cadherin–defective breast cancer. The profound antitumor effects seen in mice with E-cadherin–defective tumors suggest that the observed synthetic lethal effect might also have translational utility. Several distinct approaches could be used to assess the potential of ROS1 inhibitors in E-cadherin–defective cancers.
Figure 7. E-cadherin synthetic lethal effects operate in vivo in E-cadherin–defective breast tumors. A, Therapeutic response to foretinib treatment in mice bearing E-cadherin-deficient mammary tumors. Mammary tumor fragments from KEP mice were transplanted into 22 recipient mice; once tumors had established, animals were treated over a 27-day period with either drug vehicle or foretinib (25 or 50 mg/kg every other day, n = 8 for vehicle-treated cohorts and n = 7 for each drug treatment cohort). Tumor volumes after the initiation of treatment are shown. ANOVA P < 0.0001 for both foretinib treatment regimes compared with vehicle-treated mice. B, Kaplan–Meier plot of data from A indicating antitumor efficacy of 25 mg/kg foretinib treatment. C, Kaplan–Meier plot of data from A, indicating effect of 25 mg/kg crizotinib treatment. D, Immunohistochemistry images of tumors extracted from animals from A at the end of foretinib treatment are shown. Representative images of H&E, Ki67, and cleaved caspase-3 are shown (magnification, ×20). Scale bars, 250 μm. E, Therapeutic response to crizotinib treatment in mice bearing KEP tumor allografts as in A. ANOVA P < 0.0001 for both crizotinib treatment regimes compared with vehicle-treated mice. F, Data from E plotted to illustrate tumor volume reduction in both crizotinib-treated cohorts. G, Kaplan–Meier plot of data from E for vehicle versus crizotinib 50 mg/kg cohort. H, Immunohistochemistry images illustrating lack of E-cadherin expression in a PDX model of breast cancer (BCM2665) compared with positive [HCC1954 breast tumor cells] and negative [MDAMB231 breast tumor cells] controls. J, Therapeutic response to foretinib treatment in mice bearing BCM2665 PDX. BCM2665 was transplanted into 19 recipient mice; once tumors had established, animals were treated over a 47-day period with either drug vehicle (n = 11) or foretinib (25 mg/kg every other day, n = 8) as shown. ANOVA = P < 0.0001 K, Kaplan–Meier plot of data from J. L, Representative images of formalin-fixed, paraffin-embedded tumors from animals in I stained with H&E, Ki67, and cleaved caspase-3 are shown (magnification, ×20). Scale bars, 250 μm.
For example, clinical trials in advanced forms of ILC, where E-cadherin defects are common, might seem appropriate. In such trials, absence of E-cadherin using immunohistochemical analysis could be used to preselect appropriate patients for treatment. As crizotinib is already used in the treatment of non-small cell lung cancer and has been the subject of prior clinical safety assessment, this drug seems a reasonable candidate for testing in such trials.

Mechanistically, our data suggest that loss of E-cadherin imparts upon cells a dependency upon ROS1 that is likely related to the ability to undergo cytokinesis. Our work suggests that p120 defects could play a role in these phenotypes, but it also seems possible that other proteins contribute to the synthetic lethal phenotype. For example, we also found that other proteins associated with actomyosin control such as MRCKα are also synthetic lethal with E-cadherin defects; these could conceivably play a part in the ROS1/E-cadherin synthetic lethal effect. Although drug-like inhibitors of RHO effector kinases such as MRCKα are still in development (46), licensed drugs such as crizotinib might provide an actionable approach to targeting E-cadherin-defective cancers. Similarly, although we were able to elicit E-cadherin synthetic lethal effects with multiple different ROS1 siRNA reagents and to partially reverse crizotinib sensitivity with a p.G2032R-mutant ROS1 fusion cDNA (Fig. 2), the antitumor therapeutic effect of drugs such as crizotinib or foretinib could also be influenced by inhibition of kinases other than ROS1. Although we were unable to elicit synthetic lethality with siRNAs for MET, ALK, AXL, and KDR in isogenic or nonsisogenic systems (Supplementary Figs. S2 and S5), we cannot formally exclude the possibility that other kinase targets of foretinib or crizotinib also contribute to the E-cadherin synthetic lethality. It seems possible that clinical studies where mechanisms of resistance to crizotinib in E-cadherin-defective tumors are assessed will inform this area.

METHODS

Materials and Cell Lines

Small-molecule inhibitors were obtained from SelleckChem. siRNAs were obtained from GE Dharmacon. Cell lines were obtained from the ATCC and European Collection of Cell Cultures in 2010 and 2011 and maintained according to the supplier’s instructions, as described previously (19, 47). The MCF10A CDH1−/− and MCF10A CDH1+/+ isogenic cell lines were obtained in 2014 from Sigma Aldrich and maintained according to the supplier’s instructions. At 6-month intervals and prior to storage, the identity of each cell line was confirmed by short tandem repeat profiling of 10 loci using the GenePrint 10 system (Promega). At monthly intervals, Mycoplasma testing of cell cultures was carried out using the MycoAlert Mycoplasma Detection Kit (Lonza).

CRISPR/Cas9 Targeting

MCF7 cells were targeted using the Edit-R-CRISPR-CAS9 gene engineering kit (GE Dharmacon) according to the supplier’s instructions. The following crRNA sequence was used: 5′-GCUGAG GAUGGUGUAAAGCGAGUUUUAGAGCUAUGCUGUUUUG-3′. MCF7 cells were transfected in 24-well plates (100,000 cells/well) with tracerRNA, crRNA, and Cas9 plasmid. Seventy-two hours after transfection, cells were plated in 15-cm dishes and continuously cultured until colonies formed. Colonies were recovered and profiled using PCR and Sanger sequencing to determine the presence of CDH1 mutations.

RNAi and Small-Molecule Synthetic Lethal Screens

siRNA screens and small-molecule screens were performed as described previously (19, 48). A list of small-molecule inhibitors and their molecular targets used in the screens is shown in Supplementary Table S10. See also Supplementary Materials and Methods for details.

Cell Survival and Apoptosis Assays

Cell survival analysis was conducted as previously described (49). See also Supplementary Materials and Methods for details.

Protein Analysis

Whole-cell protein extracts were prepared from cells lysed in NP250 buffer (20 mmol/L Tris pH 7.6, 1 mmol/L EDTA, 0.5% NP40, 250 mmol/L NaCl), supplemented with protease inhibitor cocktail tablets (Roche). Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad). For Western blot analysis, 50 μg of whole cell lysates were electrophoresed on Novex 4% to 12% gradient bistris precast gels (Innogen) and immunoblotted overnight at 4°C with antibodies listed in Supplementary Table S11.

Confocal Microscopy

Cells were plated on coverslips and exposed to drugs the following day. After drug exposure, cells were fixed in 4% (v/v) paraformaldehyde for 10 minutes, washed, permeabilized in 0.2% (v/v) Triton X-100 in PBS for 20 minutes, washed, and blocked in immunofluorescence buffer IFF [1% (w/v) bovine serum albumin, 2% (v/v) PBS in PBS] then immunostained with primary antibodies targeting F-Actin conjugated to Alexa Fluor 488 and Tubulin (Santa Cruz Biotech) and detected with a Texas red conjugated secondary antibody (Supplementary Table S11). DAPI staining was used to detect nuclei. Mitotic and nuclear phenotypes of at least 200 cells per condition were scored in each replicate experiment. Confocal experiments were imaged using Zeiss CLM700.

Time Lapse Microscopy

Time-lapse microscopy was performed in 6-well plates using a Diaphot inverted microscope (Nikon), in a humidified CO₂ chamber at 37°C, using a motorized stage (Prior Scientific), controlled by Simple PCI software (Compix). Cells were first transfected with a mCherry-H2B plasmid, FACSort for mCherry-H2B to facilitate DNA visualization, and then exposed to foretinib or crizotinib for a 24-hour period, over which they were microscopically imaged. Sorting of cells is described in Supplementary Materials and Methods.

Cell-Cycle Analysis

Measurements took place on a BD LSR II SORP flow cytometer (BD Biosciences) equipped with a 488-nm blue laser, a 561-nm yellow laser, a 633-nm red laser, and a 405-nm violet laser. Propidium iodide was measured with 600 LP 610/20 BP. Cell population was gated in a FSC/SSC dot plot and doublets were gated out based on a DNA dye area/width dot plot. This cell population was further analyzed regarding its cell distribution (G1, S, and G2/M phase) using FlowJo V software.

In Vivo Assessment of Foretinib and Crizotinib Efficacy

In vivo efficacy studies were conducted using KEP or BCM2665 PDX tumor-bearing mice as previously described (45, 50). Experiments...
on KEP tumor-bearing mice were carried out at the Netherlands Cancer Institute according to local and international regulations and ethical guidelines and were approved by the local animal experimental committee at the Netherlands Cancer Institute (DEC-NKI AvD:30100 2015 407 Appendix I, WP 5791 and WP 5900). BALB/c nude mice were implanted with KEP tumor sections in their fourth mammary gland on the right side as described previously (50). Once tumors reached 200 mm³ in volume, mice were randomized into cohorts who received either crizotinib/foretinib 25 or 50 mg/kg a day via oral gavage for 28 days or the drug vehicle. Operators were blinded to which cohort received foretinib or crizotinib and which received vehicle. A surrogate of animal survival was used and defined by the amount of time taken for tumors to reach a pre-agreed volume of 1,500 mm³, at which point mice were sacrificed. Experiments using the BCM 2665 PDX model were conducted at the Institute of Cancer Research (ICR) according to local (UK Home Office) and international regulations and ethical guidelines and were approved by the local animal experimental committee at the ICR. Viably frozen fragments of the BCM2665 model (45) were obtained from Dr. Michael Lewis, Baylor College of Medicine, Houston, Texas, under material transfer agreement and propagated in SCID-Beige and NSG hosts. SCID-beige host mice were obtained from Charles River Laboratories at 21 to 28 days of age and were implanted with BCM2665 tumor fragments (~2 mm in length) subcutaneously into the inguinal mammary fat pad following standard procedures. Once tumors measured 2 mm in diameter (assessed by palpation and caliper measurement), mice were randomized into cohorts who received either foretinib (25 mg/kg) or the drug vehicle (1% hydroxypropylmethylcellulose, 0.2% SDS in H₂O) every day (four days per week) by oral gavage. Operators were blinded to which cohort received foretinib and which received vehicle. Tumor growth was monitored over time (assessed by palpation and caliper measurement) and a surrogate of animal survival defined by the amount of time taken for tumors to reach a pre-agreed 12-mm diameter, at which point mice were sacrificed and tumors excised. Tumor volume was calculated using the formula V = (π × length × width²/6), where the length is the largest tumor diameter and width is the perpendicular diameter. Statistical analysis was performed using Prism. Tumors were formalin-fixed and paraffin-embedded, and slides were stained with H&E, or immunohistochemistry with antibodies against Ki67 and cleaved caspase-3 was undertaken using standard procedures.

Disclosure of Potential Conflicts of Interest

M. Dowsett reports receiving commercial research grants from Pfizer and Radius, has received honoraria from the speakers bureau of Roche, is a consultant/advisory board member for Radius, and has received remuneration from ICR Rewards for Inventors. No potential conflicts of interest were disclosed by the other authors.

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