Immunotherapy Targeting Folate Receptor Induces Cell Death Associated with Autophagy in Ovarian Cancer

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Abstract

Purpose: Cancer cells are highly dependent on folate metabolism, making them susceptible to drugs that inhibit folate receptor activities. Targeting overexpressed folate receptor alpha (FRA) in cancer cells offers a therapeutic opportunity. We investigated the functional mechanisms of MORAB-003 (farletuzumab), a humanized mAb against FRA, in ovarian cancer models.

Experimental Design: We first examined FRA expression in an array of human ovarian cancer cell lines and then assessed the in vivo effect of MORAB-003 on tumor growth and progression in several orthotopic mouse models of ovarian cancer derived from these cell lines. Molecular mechanisms of tumor cell death induced by MORAB-003 were investigated by cDNA and protein expression profiling analysis. Mechanistic studies were performed to determine the role of autophagy in MORAB-003–induced cell death.

Results: MORAB-003 significantly decreased tumor growth in the high-FRA IGROV1 and SKOV3ip1 models but not in the low-FRA A2780 model. MORAB-003 reduced proliferation, but had no significant effect on apoptosis. Protein expression and cDNA microarray analyses showed that MORAB-003 regulated an array of autophagy-related genes. It also significantly increased expression of LC3 isofrom II and enriched autophagic vacuolization. Blocking autophagy with hydroxychloroquine or bafilomycin A1 reversed the growth inhibition induced by MORAB-003. In addition, alteration of FOLR1 gene copy number significantly correlated with shorter disease-free survival in patients with ovarian serous cancer.

Conclusions: MORAB-003 displays prominent antitumor activity in ovarian cancer models expressing FRA at high levels. Blockade of folate receptor by MORAB-003 induced sustained autophagy and suppressed cell proliferation. Clin Cancer Res; 21(2); 448–59. ©2014 AACR.

Introduction

The folate receptor 1 gene (FOLR1) encodes a 38-kDa GPI-anchored protein (folate receptor alpha, or FRA) that binds folic acid with high affinity and transports folate by receptor-mediated endocytosis (1). FRA is selectively overexpressed in approximately 90% of nonmucinous ovarian carcinomas, but is largely absent from normal tissues (2, 3). Its expression is maintained on metastatic foci and recurrent tumors (4). Studies that have examined the functional significance of FRα in ovarian cancer cells have shown that higher receptor expression is associated with greater biologic aggressiveness (5).

Previous studies showed that anti-FRα antibodies such as LK26, Mov18, and Mov19 have reached development stages and been tested for clinical relevance (6, 7). MORAB-003 (farletuzumab), which is a humanized anti-FRα mAb derived from optimization of the LK26 molecule; it maintains an affinity similar to the original murine LK26 antibody (approximately 2 nmol/L) and a tissue-binding profile consistent with FRα distribution (5, 8). It has been shown to inhibit cell growth and activate antibody-dependent, cell-mediated cytolysis (5, 8, 9). Although MORAB-003 has been shown to act through antibody-dependent cellular cytolysis in vitro (10), additional mechanisms underlying its function are likely. Herein, we reported that sustained treatment with MORAB-003 antibody in xenograft models of human ovarian cancers or in three-dimensional (3D)–cultured ovarian cancer models expressing high levels of FRα induced enrichment of late-stage autophagic events, eventually leading to cell death and tumor growth inhibition.

Materials and Methods

Cell lines and cultures

Human ovarian cancer cells (A2780, HeyA8, SKOV3ip1, and IGROV1) were cultured in RPMI-1640 cell culture medium
supplemented with 15% FBS and 0.5% gentamicin (11, 12). All cell lines were purchased from the ATCC and authenticated by the Cell Line Core Facility at The University of Texas MD Anderson Cancer Center and routinely tested to confirm the absence of Mycoplasma. For the 3D models, cells were grown on nonadhesive optical plates (MatTek Corporation) and then brought to the glass surface, which was precoated with 200 μL of growth factor-reduced Matrigel (BD Biosciences). Approximately 18,000 to 20,000 spheroid-like cells were plated in each well of a 24-well plate and incubated at 37°C for 1 hour to allow cells to seed gradually. Spheroids were cultured with full medium containing 2% Matrigel (13). The culture conditions and growth of 3D spheroids were monitored daily in preparation for drug treatment.

Reagents and transfection

The pcDNA–RFP–LC3 plasmid in lentiviral vector was derived from Addgene plasmid 21074 (Addgene; ref. 14). The PEA-15 siRNA (ggaagacauccucagcaagg) was described previously (15). The EGFP–LC3 plasmid was derived from Addgene 11546 (16). The siRNAs were applied to cells at a final concentration of 100 nmol/L for 48 hours. The shRNAs against BECN-1, 29-mer mix (human) were purchased from Origene, Inc. Transfections were performed according to the manufacturer’s instructions.

Analysis of FRα expression

Cellular expression of FRα was determined by FACS analysis of cells stained with fluorescein isothiocyanate–labeled MORAB-003. The mean fluorescence value was 1 in cells that do not express FRα, such as CHO-parental cells. The expression of FRα was validated with FRα antibody PAS-27465 (Thermo Scientific Pierce).

Animals

The female athymic nude mice used for this experiment were cared for according to guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the U.S. Public Health Service policy on Humane Care and Use of Laboratory Animals. All mouse studies were approved and supervised by The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee. Development and characterization of the orthotopic mouse models of advanced ovarian cancer have been described previously (11, 17).

In vivo MORAB-003 therapeutic dose finding

To determine the optimal dose and schedule of MORAB-003 therapy, 5 mice were injected i.p. with SKOV3ip1 cells to induce tumors. One week later, treatment was initiated with either control IgG or MORAB-003 (5, 25, or 50 mg/kg) twice or thrice weekly for 28 days. Mean tumor weights were recorded after 4 weeks of therapy for each treatment group.

In vivo murine ovarian cancer models

Mice were injected with tumor cells [1 × 106 cells/ml (SKOV3ip1 and A2780) or 2 × 106 cells/ml (IGROV1) in Hank balanced salt solution] to induce tumors; 1 week later, tumor-bearing mice were randomized into four groups (n = 10 mice/group for SKOV3ip1 or IGROV1 models, and n = 5 for A2780 model or control) and treated with i.p. injections of the following agents: (i) control IgG (5.0 mg/kg twice weekly), (ii) MORAB-003 (5.0 mg/kg twice weekly), (iii) control IgG (5.0 mg/kg twice weekly) plus docetaxel (35 μg/mouse weekly), or (iv) MORAB-003 (5.0 mg/kg twice weekly) plus docetaxel (35 μg/mouse weekly). Mice were monitored daily for adverse effects and killed if they became moribund. After 4 to 5 weeks of therapy, all of the mice were killed and necropsies were performed. For each mouse, body and tumor weight, tumor distribution, number of tumor nodules, and amount of ascites were recorded at necropsy. Tissue specimens were fixed in formalin for paraffin embedding or snap frozen in optimal cutting medium (Miles, Inc.) for frozen slide preparation.

Tandem mRFP/mGFP-LC3 fluorescence microscopy

pGFP–RFP–LC3 (Addgene plasmids (14) or EGFP–LC3 plasmids (16) were stably transfected into epithelial ovarian cancer cells (HeyA8 and SKOV3). After 3D culture on glass-bottom optical 35-mm2 dishes (MatTek Corporation), expression of EGFP and RFP was visualized with a laser scanning multiphoton confocal microscope (TCS SPS MP; Leica).

Apoptosis assay

Apoptosis was measured by using the Annexin V–7AAD Apoptosis Detection Kit I (BD Biosciences). Twenty-four hours and 7 days following treatment with MORAB-003, SKOV3ip1 cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1 × 106 cells/mL. Solution of 100 μL (1 × 105 cells) was then transferred to a 5-mL culture tube. Annexin V and 7-AAD (5 μL each) were added to the solution. Cells were subjected to gentle vortexing and then incubated for 15 minutes at room temperature (25°C) in the dark. A 1× binding buffer (400 μL) was added to each tube. Samples were analyzed by flow cytometry within 1 hour.

Immunohistochemistry for PCNA and CD31 and TUNEL assay

Formalin-fixed, paraffin-embedded tumor tissues collected from the mice after treatment were sectioned (5 μm) for detection of PCNA (proliferating cell nuclear antigen) and for TUNEL (terminal deoxynucleotidyl transferase-mediated nick-end labeling) assay according to the previous protocol (18). For quantification of PCNA, microvessel density (MVD) or TUNEL...
expression, the numbers of PCNA- or TUNEL-positive tumor cells or CD31-positive endothelial cells were counted in 10 random fields at ×200 magnification.

**Immunoblotting**

Immunoblotting analyses were performed as previously described (11).

**Cell viability and proliferation**

To determine the *in vitro* effects of MORAB-003 on ovarian cancer cell viability, SKOV3ip1 cells were cultured in folate-free medium supplemented with 10% dialyzed FBS and 0.1% gentamicin sulfate for 5 days. Cells were then plated in medium supplemented with 5-MTF (i.e., physiologic folate conditions), based on the previous protocol (19). Experimental conditions were set in triplicate.

To assess cell viability, 50 μL per well of 0.15% MTT (Sigma) was added a 96-well plate, and the plate was incubated for 2 hours at 37°C. The medium/MTT preparation was then removed from each well, and 100 μL of DMSO (Sigma) was added. After incubation for 20 minutes, absorbance at 570 nm was read (Ceres UV 900C; Bio-Tek Instrument, Inc.) within 30 minutes. Each treatment was repeated in 8 individual replicas.

To assess *in vitro* cell proliferation, the percentages of proliferative cells were measured by using Click-IT EdU Flow Cytometry Assay Kits (Invitrogen) according to the manufacturer’s protocol. The two-dimensional (2D)—cultured cells were incubated with 10 mmol/L EdU (5-ethyl-2'-deoxyuridine) for 2 hours and the trypticized cells fixed with 4% paraformaldehyde before staining and flow cytometry. For 3D-cultured spheroids, cells were extracted with Accutase solution (Sigma) to dissociate each well, and 100 μL of DMSO (Sigma) was added. After incubation for 20 minutes, absorbance at 570 nm was read (Ceres UV 900C; Bio-Tek Instrument, Inc.) within 30 minutes. Each treatment was repeated in 8 individual replicas.

**Acridine orange staining and flow cytometry**

The formation of acidic vesicular organelles (AVO) was visualized by acridine orange (AO; ref. 20). In AO-stained cells, the cytoplasm and nucleolus fluorescence bright green and dim red, whereas acidic compartments fluoresce bright red. The volume of the cellular acidic compartment can be quantified, therefore, by the intensity of the red fluorescence. Cells were stained with AO as described previously (20). Green (510–530 nm) and red (>650 nm) fluorescence emissions from 1 × 10⁴ cells illuminated with blue (488 nm) excitation light were measured with a FACSCalibur from Becton Dickinson and analyzed by CellQuest software (BD Biosciences).

**Transmission electronic microscopy**

3D-cultured SKOV3ip1 spheroids were fixed and processed as previously described (21).

**Reverse-phase protein arrays**

Protein lysates were extracted and submitted to the RPPA Process Core Facility at MD Anderson Cancer Center (http://bioinformatics.mdanderson.org/OOMPA; ref. 22). Slides were probed with 263 validated antibodies, then were scanned, and analyzed to quantitatively measure spot density to generate a fitted curve for each condition. We included only the data for the 172 individual antibodies with QC Scores higher than 0.80 in the heatmaps for the SKOV3ip1 array and the 214 antibodies higher than 0.80 for the IGROV1 array. The fitted curve was plotted with the log, concentration of proteins versus spot density. All data presented reflect fold change compared with the baseline (i.e., to control-treated cells). Positive fold change was calculated by dividing each linear value greater than 1 by the average control linear value for each antibody tested, whereas negative fold change (for linear values <1.0) was also calculated [by using the following formula: (−1/linear fold-change)] and plotted in a heat map as a log 2.0 value. Original data for reverse-phase protein array (RPPA) were provided in Supplementary Table S1.

**Microarray analysis**

SKOV3ip1 3D spheroids with or without MORAB-003 treatment were subjected to microarray analysis under the following conditions as previously reported (19): (i) cells plated in folate-free medium (7 days), (ii) cells plated in medium with physiologic levels of folic acid (2–10 nm; 7 days), (iii) cells plated with physiologic levels of folic acid (7 days) and MORAB-003 (24 hours), and (iv) cells plated with physiologic levels of folic acid (7 days) and control antibody (24 hours). For each condition, three aliquots from each RNA sample were then hybridized to Affymetrix U133 2 arrays, for a total of 15 arrays. Integrated pathway analysis networks were generated by using Core analysis with indicated data cutoffs for up/downregulated genes and using direct interactions, with the cutoff for network size at 70. The highest scoring five networks were merged and exported as IPEG files. All network plottings were carried out according to the method described previously (23). The original microarray data were uploaded to GEO with accession number GSE61513 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61513).

The Cancer Genome Atlas database analysis

Data from The Cancer Genome Atlas (TCGA) were evaluated for expression of the FOLR1 gene in samples from patients with ovarian serous cystadenocarcinoma and expression of cluster immunoreactive signatures (n = 110; TCGA) in relation to the patients’ overall survival and disease-free survival (DFS). We also queried data from patients with uterine corpus endometrial carcinoma (n = 82; www.cbioportal.org; refs. 24, 25).

**Statistical analysis**

All results are expressed as mean ± SD unless indicated otherwise. Continuous variables were compared by using the Student t test (between two groups) or ANOVA (for all groups). For non-normally distributed datasets (as determined by the Kolmogrov–Smirnov test), the Mann–Whitney rank-sum test was used. A P < 0.05 was considered statistically significant. At least three independent replicates were included for each test.

**Results**

**In vivo antitumor effects of MORAB-003 in human ovarian cancer**

We first characterized FRα expression of human ovarian cancer cell lines by using FACS analysis. Compared with a mean fluorescence value of 1 in cells that do not express FRα (CHO parental cells, A2780 or HeyA8 cells), FRα expression was 602-fold higher in the transfected cell line CHO-FRα, 170-fold higher in IGROV1 cells, and 6.2-fold higher in SKOV3ip1 cells (Fig. 1A). We
confirmed the expression of FRα in an array of ovarian cancer cell lines by Western blots with FRα antibody (Supplementary Fig. S1A). We also determined the basal levels of autophagic flux as measured by isoform II of the microtubule-associated protein 1 light chain (LC3II), but did not observe significant differences across the cell lines regardless of levels of FRα.

We first performed a dose-finding experiment using the SKOV3ip1 orthotopic mouse model to determine the optimal dosage. A dose of 5 mg/kg twice weekly was chosen (Supplementary Fig. S1B), as no additional benefit was noted at higher doses; this dose is within clinically relevant doses (26). We then tested the therapeutic effect of MORAB-003 in orthotopic mouse models of IGROV1, SKOV3ip1, and A2780 tumors. Because taxanes are frequently used for treatment of newly diagnosed or relapsed ovarian cancer, we also tested combinations of MORAB-003 with docetaxel. Compared with the control, MORAB-003 monotherapy reduced tumor weight by 44% in the SKOV3ip1 model (P < 0.05) and 84% in the IGROV1 model (P < 0.05; Fig. 1B). Furthermore, the combination of MORAB-003 and docetaxel significantly reduced tumor weight in both SKOV3ip1 and IGROV1 tumor-bearing mice compared with docetaxel monotherapy. MORAB-003 monotherapy also reduced the numbers of tumor nodules in the SKOV3ip1 and IGROV1 models in comparison with the control group (P < 0.05; Supplementary Fig. S1C and S1D). However, neither tumor weight (Fig. 1C) nor number of tumor nodules was reduced by MORAB-003 treatment in the A2780 model (Supplementary Fig. S1E). In all of these experiments, there was no significant difference in feeding behavior or average mouse weight among the various treatment groups, suggesting that MORAB-003 has no systemic toxicity (Supplementary Fig. S1F–S1H).

We then examined tumor sections from different treatment groups for markers of proliferation (PCNA), apoptosis (TUNEL), and angiogenesis (CD31). Treatment with MORAB-003 reduced tumor cell proliferation by 20% to 27% in comparison with untreated controls (P < 0.05), but had no significant effects on angiogenesis or tumor cell apoptosis (Fig. 2A).

Blocking FRα with MORAB-003 induces sustained autophagy To recapitulate the in vivo inhibitory effects of MORAB-003, we performed in vitro analyses in both 2D-cultured monolayer SKOV3ip1 cells and 3D-cultured SKOV3ip1 spheroid-like models (27). An EdU incorporation assay showed that MORAB-003 (5 μg/mL) treatment decreased proliferation of 3D-cultured SKOV3ip1 spheroids by approximately 40% in comparison with untreated controls (P < 0.001; Fig. 2B), whereas it decreased proliferation of 2D-cultured SKOV3ip1 cells by only approximately 18% (Fig. 2B, P < 0.001). Given the substantial difference between the effects of MORAB-003 in 2D and 3D conditions, we reasoned that the in vivo inhibitory mechanism of MORAB-003 may be recapitulated by 3D-cultured cancer spheroids (28), as shown by representative images for spheroids treated with IgG or MORAB-003 (Fig. 2B).

To determine whether the inhibitory mechanism of MORAB-003 is related to apoptosis, we first performed FACS analysis with Annexin V and propidium iodide (PI) staining in 3D SKOV3ip1 spheroids. We observed no significant increase in cell apoptosis in treated cells, particularly late-stage apoptosis (Annexin V*PI+). The percentage of Annexin V*PI+ cells in the cells treated with control IgG (9.28% ± 0.98) was not significantly different than that in cells treated with MORAB-003 (12.0% ± 0.25; Supplementary Fig. S2A and S2B). This indicates that the inhibitory effect of MORAB-003 on cell proliferation of 3D SKOV3ip1 spheroids is likely not mediated through an apoptotic mechanism. We then tested various doses of MORAB-003 on the viability of cells in 3D SKOV3ip1 spheroids and found that 5 μg/mL of MORAB-003 significantly reduced viability compared with controls (P < 0.001; Supplementary Fig. S2C).

Next, RPPA analysis was performed to determine the protein expression profile of SKOV3ip1 tumors. A number of autophagy...
Figure 2.
Effects of MORAB-003 in vivo or in 3D ovarian cancer spheroids and 2D monolayer–cultured ovarian cancer cells. A, in the in vivo IGROV1 tumor model, treatment with MORAB-003 resulted in a 27% decrease in tumor cell proliferation by PCNA staining; \( P < 0.001 \) (control vs. MORAB-003). However, MORAB-003 treatment did not significantly affect angiogenesis (i.e., MVD), as detected by CD31 staining, or tumor cell apoptosis, as detected by TUNEL assay. B, left, treatment with MORAB-003 decreased cell proliferation (as measured by EdU incorporation) by 20% in 2D-cultured SKOV3ip1 cells and by 80% in 3D SKOV3ip1 spheroids \( P < 0.001 \) (control vs. MORAB-003). Right, representative images show 3D SKOV3ip1 spheroids before treatment or after treatment with control IgG or MORAB-003 antibody for 72 hours. C, left, heatmap clustered from RPPA analysis in tumor samples of the SKOV3ip1 model revealed that an array of autophagic factors, including mTOR (marked by asterisk) and RICTOR, were downregulated by MORAB-003. Right, other autophagic factors, including S116-PEA-15 and beclin-1 (marked by asterisks), were upregulated by MORAB-003 treatment. D, normalized fold changes in Z-score indicate that mTOR, S116-PEA-15, and beclin-1 were significantly regulated by MORAB-003 treatment. E, left, network pathway analysis in SKOV3ip1 3D spheroids treated with control antibody or MORAB-003. Different colors represent different interactions: red, gene regulation; green, reactome interaction. Right, Western blots show that treatment of 3D SKOV3ip1 spheroids with MORAB-003 led to elevated levels of ATG4B, ATG3, an ATG14-containing PIK3C3 complex with phosphorylated PIK3C3 (S164), and ATG14. Level of unphosphorylated PIK3C3 was decreased upon MORAB-003 treatment. Fold density is shown at the top of each, and control IgG was normalized as 1.
factors were regulated by treatment with 5 μg/mL of MORAB-003, including mTOR and rapamycin-insensitive companion of mTOR (RICTOR), were downregulated (Fig. 2C), and S116-phosphorylated phosphoprotein enriched in astrocytes (S116-PEA-15; a factor involved in activation of autophagic cell death in cancer cells; ref. 29), and beclin-1 (central mediator for formation of autophagosomes; Fig. 2C), were upregulated. RICTOR and mTOR are known to activate autophagy in response to environmental stress (30, 31). Levels of beclin-1 (32) and S116-PEA-15 were significantly increased (by >2-fold) by MORAB-003 treatment (Fig. 2D). The same screening by RPPA was performed in IGROV1 tumors, and the results also indicated that S116-PEA-15 and beclin-1 were upregulated by MORAB-003 treatment (Supplementary Fig. S3A).

We then analyzed the transcriptional profiles of SKOV3ip1 tumor cells using a cDNA microarray. Using a data-biased network analysis (23), we found that treatment with MORAB-003 significantly upregulated a number of genes involved in autophagy initiation and progress, including LC3II (MAP1LC3B), ATG3, ATG4B, and BECN1 (Fig. 2E, left), supporting the findings of the RPPA. To further confirm the regulation of autophagy signaling by MORAB-003, we determined the levels of ATG4B, ATG3, S164-phosphorylated PIK3C3, and its partner ATG14 (33), in 3D-cultured SKOV3ip1 spheroids. We found that these signaling cascades were upregulated by MORAB-003 treatment in comparison with controls (Fig. 2E, right). We also found that MORAB-003 treatment slightly decreased levels of the PIK3C3 complex.

So far, little is known about the phosphorylation status of PEA-15 at Ser116 in tumors or the impact of this phosphorylation on the function of PEA-15 during tumor progression. Our studies discovered that S116-PEA-15 may play a tumor-suppressive role during MORAB-003–induced autophagy. This finding was validated by Western blot analysis of lysates of SKOV3ip1 tumors, showing that the tumors of mice treated with MORAB-003 had significantly greater levels of S116-PEA-15 and beclin-1 and lower levels of mTOR than tumors from mice treated with IgG (Fig. 3A). Next, we examined the dynamic regulation of S116-PEA-15 during MORAB-003–induced autophagy. Accumulation of S116-PEA-15, rather than total PEA-15, reached its highest levels following treatment with MORAB-003 in 3D SKOV3ip1 spheroids but not in A2780 spheroids. This finding indicated a role for activated S116-PEA-15 during MORAB-003–induced autophagy (Fig. 3B). We validated this finding in the IGROV1 3D spheroids and found that knockdown of PEA-15 with siRNA blocked the autophagic flux, as measured by conversion of LC3I to LC3II and elevation of cleaved caspase-3 level (Supplementary Fig. S3B.) This indicated that MORAB-003–induced autophagy contributes to caspase-3–activated cell death.

Next, we determined the expression of selected autophagic markers, including beclin-1 and LC3II, in sections of SKOV3ip1 and A2780 tumors from the mouse models. Treatment with
Figure 4. Induction of sustained autophagy by MORAB-003. A, top, a representative FACS plot shows the percentage of AVOs, that is, late-stage autophagosomes, as detected by AO staining, in SKOV3ip1 cells treated with control (CTL), MORAB-003 alone, docetaxel, or a combination of MORAB-003 with docetaxel. Bottom, the percentage of AVOs in A2780 and SKOV3 cells treated with MORAB-003 alone or in combination with docetaxel. (Continued on the following page.)
MORAB-003 raised the levels of beclin-1 substantially in the SKOV3ip1 tumors (Fig. 3C). Levels of LC3I, a marker for autophagic processing (16), were also elevated in SKOV3ip1 tumors treated with MORAB-003 (Fig. 3D). However, no increases of beclin-1 or LC3II levels were observed in the low-FrxC A2780 tumors (Fig. 3C and D). We also examined the role of beclin-1 in MORAB-003–induced autophagy in vitro in the 3D-cultured IGROV1 cells. Knockdown of beclin-1 in these cells by an shRNA complex against BECN1 (Supplementary Fig. S3C) blocked the accumulation of LC3I and abolished the MORAB-003–induced autophagic flux. Knockdown of beclin-1 also reduced the MORAB-003–induced elevations in cleaved caspase-3 levels (Supplementary Fig. S3D). This result indicated that beclin-1 mediates MORAB-003–induced autophagy and plays a role in crosstalk for this autophagy with caspase-3–activated cell death.

Third, we quantitatively measured the accumulation of AVOs in cytoplasm using FACs with AO staining (34). Treatment with MORAB-003, alone or in combination with docetaxel, significantly raised the percentages of AVOs (i.e., the AO+ population) in 3D SKOV3ip1 spheroids (P < 0.001, MORAB-003 vs. controls; P < 0.001, MORAB-003 + docetaxel vs. controls; Fig. 4A). However, MORAB-003 did not significantly increase the percentage of AVOs in 3D A2780 spheroids (P > 0.5, MORAB-003 vs. controls; Fig. 4A). We then determined the levels of autophagic markers, including SQSTM1/p62 (35), beclin-1, and conversion of LC3I to LC3II, in A2780, and SKOV3ip1 3D spheroids by Western blot analysis. MORAB-003 treatment increased levels of beclin-1 and LC3II (converted from LC3I) in SKOV3ip1 cells but not in A2780 cells (Fig. 4B). The densitometry of beclin-1 and LC3II (converted from LC3I) in these blots was analyzed by ImageJ software (NIH). MORAB-003 treatment decreased the level of SQSTM1/p62, a specific autophagy substrate during autophagy maturation (36, 37), in the SKOV3ip1 model but not in the A2780 model. This indicates that a possibility that ubiquitin protein degradation processes linked with lysosome fusion were initiated by MORAB-003.

We then performed real-time videomicroscopy of 3D SKOV3ip1 spheroids expressing a monomeric pGFP–RFP–LC3 plasmid (14, 38) to dynamically visualize LC3-labeled cytoplasmic autolysosomes. We used this method to monitor autophagic flux. To determine whether MORAB-003–induced autophagy links with cell death, we used inhibitors of late-stage autophagy, including baflomycin A1 (39) or hydroxchloroquine (HCQ; ref. 40), to block the autophagic vacuolization and fusion. We found that both baflomycin A1 and HCQ reversed the MORAB-003–induced reduction of cell viability in the SKOV3ip1 3D spheroids (Fig. 4D). Addition of baflomycin A1 to MORAB-003 or a single treatment with baflomycin A1 decreased the percentages of AVOs in SKOV3ip1 3D spheroids, indicating the blockade of MORAB-003–induced autophagosome formation (Fig. 4E). Addition of HCQ to MORAB-003 also decreased the percentages of AVOs in comparison with HCQ or MORAB-003 alone, possibly because accumulation of late-stage autolysosomes induced by MORAB-003 was blocked by HCQ through inhibition of lysosome acidification (Fig. 4E). Together, these results in 3D cancer spheroids suggest that MORAB-003 treatment has potential for inhibiting tumor cell growth by sustaining late-stage autophagy.

We then used transmission electron microscopy to analyze vacuole localization in SKOV3ip1 3D spheroids. MORAB-003 induced large amounts of autophagosomes (also called early autophagosomes). Inhibition of Ovarian Cancer Growth by MORAB-003

(Continued) Only in SKOV3ip1 cells, which express high levels of Frx, did MORAB-003 induce prominent increases in the AO+ population, which as late-stage autophagosomes lead to programmed cell death P < 0.001 (control vs. MORAB-003), B. expression of factors for autophagic processing, including SQSTM1/p62, conversion of LC3I to LC3II, and beclin-1, was measured by immunoblotting lysates from 3D-cultured A2780 and SKOV3ip1 cells. The upregulation of autophagy was validated by elevated levels of beclin-1 and LC3II in SKOV3ip1 cells but not in A2780 cells. β-Actin was applied as loading control. The densitometry of beclin-1 (normalized to β-actin) and LC3II (converted from LC3I) in each condition was determined by ImageJ software. C. autophagic flux in 3D SKOV3ip1 spheroids was observed by real-time videomicroscopy. 3D SKOV3ip1 spheroids expressing pGFP–RFP–LC3 were treated with control IgG or MORAB-003 and the accumulation of late-stage autolysosomes was monitored by quantifying RFP–LC3 vesicles. Representative photomicrographs indicate the effect of IgG (top) or MORAB-003 (bottom) at approximate elapsed time of 0, 150, 300, 900, and 1,500 minutes, showing the conversion of GFP+RFP−LC3 to RFP+GFP−LC3 in MORAB-003–treated spheroids and the destabilized GFP−LC3 in IgG-treated spheroids; scale bar, 10 μm for all images. Larger magnified fields and high-resolution images are provided in Supplementary Fig. S9A. D, the cytotoxicity of MORAB-003 was assessed by MIT assay in SKOV3ip1 spheroids cultured under 3D conditions. After cells were exposed to physiologic levels of folic acid (7 days), they were treated with control IgG (72 hours), MORAB-003 (5 μg/mL, 72 hours), baflomycin A1 (0.16 nmol/L, 24 hours) plus MORAB-003 (72 hours), HCQ (1 nmol/L, 24 hours) plus MORAB-003 (72 hours), HCQ alone (1 nmol/L, 24 hours), or baflomycin A1 alone (0.16 nmol/L, 24 hours). Differences between groups were compared by the unpaired t test. Values are mean ± SEM (n = 6). E, the percentage of AVOs (measured by FACs with AO staining) in each group.
autophagic compartments), containing morphologically intact cytosol or organelles (shown by red arrows, Fig. 5A), and late-stage autophagy vacuoles, or autolysosomes, containing partially degraded cytoplasmic as well as organelle material (shown by yellow arrows, Fig. 5A). Furthermore, the MORAB-003–treated SKOV3ip1 spheroids had intact nuclear membranes and no
apoptosomes (Fig. 5A). We did not observe the same amounts of autophagosomes or autolysosomes in spheroids treated with IgG (Fig. 5B). Vacular localization in SKOV3ip1 spheroids was quantified in 15 individual cells in three different sections, and the numbers of autophagosomes and autolysosomes in MORAB-003–treated cells were significantly higher than in controls (P < 0.05, Fig. 5C).

**Association of FOLR1 with clinical outcome in human ovarian cancer**

Given the paucity of data regarding the association between clinical outcome and FRα expression, we analyzed data from TCGA (24) to determine the correlation between copy numbers of the FOLR1 gene and survival in patients with serous ovarian cystoadenocarcinoma. For patients with this immunoreactive tumor type, the Kaplan–Meier estimate for DFS was significantly shorter in those whose tumor had an altered copy number of the FOLR1 gene (n = 6) than in those whose tumor expressed the unaltered FOLR1 gene (Fig. 6A and B, n = 83, P = 0.039, www.cbioportal.org; ref. 24). We also examined FOLR1 gene expression in data from the Broad Institute’s CCLE cell lines. The epithelial cancer cells with the highest FOLR1 mRNA expression are endometrial and ovarian (Supplementary Fig. S4C). We then queried for alterations of FOLR1 gene copy number in a set of patients with uterine corpus endometrial carcinoma (www.cbioportal.org; TCGA; ref. 25). As in the ovarian cancer analysis, patients whose tumor had an altered FOLR1 gene copy number had a significantly shorter life span than those whose tumor expressed unaltered FOLR1 (P = 0.001855; Supplementary Fig. S4D). These data point to the potential clinical importance of alteration of FOLR1 gene copy number during development of serous ovarian cancer.

**Discussion**

In this study, we discovered that MORAB-003, a humanized mAb against FRα, significantly reduced growth of human ovarian cancers in xenograft mouse models. Specifically, MORAB-003 significantly decreased tumor growth in the high-FRα IGROV1 and SKOV3ip1 models but not in the low-FRα A2780 model, and these effects were not related to apoptosis. MORAB-003 regulated an array of autophagy-related genes and significantly increased expression of LC3 isoform II and enriched autophagic vacuolization. Blocking autophagy with an inhibitor (HCQ or bafilomycin A1) reversed the growth inhibition induced by MORAB-003. These findings constitute in vivo and in vitro evidence that MORAB-003 induced sustained autophagy in SKOV3ip1 tumors, leading to growth inhibition.

Miotti and colleagues (3) demonstrated that FRα modulated activities of signaling molecules, including lyn kinase and G protein subunits. They showed that the active (phosphorylated) form of lyn kinase associated with FRα was translocated out of lipid rafts when FRα was inhibited by LK26 (precursor of MORAB-003) or the rate of cell division was reduced by overconfluence (3). These results suggest a role for FRα in intracellular signaling associated with tumor cell proliferation. Our study provides a new understanding of the in vivo effects of FRα inhibition (i.e., by MORAB-003) on ovarian cancer. The level of FRα expression was correlated with the extent of MORAB-003 therapeutic effect. We also discovered a correlation between level of FRα and the inhibitory effect of MORAB-003. These findings are consistent with previous studies showing that FRα confers a growth advantage to cells (19, 41, 42).

Our results further point to a previously unrecognized mechanism whereby blockade of FRα by MORAB-003 inhibits tumor growth via induction of cell death associated with sustained autophagy in FRα-positive cells. Typically induced by prolonged stress, sustained autophagy eventually leads to cell death when protein and organelle turnover overwhelm the capacity of the cell (21). It interrupts the metabolic system in proliferative tumor cells, serving as an alternative or complementary mechanism to tumor suppression through induction of type II programmed cell death (43, 44). This cell growth inhibition via sustained autophagy mediated by FRα inhibition is a novel mechanism for cell death (21) and an important addition to the previous reports on cell-cycle– and apoptosis-regulatory mechanisms, including inhibition of PI3K and Akt during antibody-mediated tumor inhibition by trastuzumab (Herceptin; ref. 45) and the immune effects of rituximab (46). Our studies also showed that MORAB-003 significantly augmented the antitumor effect of docetaxel through an autophagy-associated mechanism. Given that lysosomotropic agents such as HCQ and chloroquine have been reported widely to inhibit autophagy in cancer treatment (47), this study provides new insight into the use of combinations of taxane-based regimens with MORAB-003 in improving therapeutic efficacy for ovarian cancer through induction of late-stage autophagy. The finding of association between autophagy induced by MORAB-003 and tumor growth inhibition is consistent with previous reports in which such autophagic events eventually lead to type II programmed cell death and growth inhibition in tumor cells (48–50). In addition, PEA-15 is an acidic, serine-phosphorylated phosphoprotein and an endogenous substrate
for calcium-calmodulin-dependent protein kinase II at ser116 position (51). However, the role of phosphorylation on PEA-15 that switches it from tumor suppressor to tumor promoter remains controversial. Our studies indicate that S116-PEA-15 plays a critical role during MORAB-003–induced autophagy that leads to cell death. Further studies are needed to characterize the roles of S116-PEA-15, S164-PIK3C3, and ATG14 complex in ovarian cancer during MORAB-003–mediated induced autophagy.

In summary, our findings indicate that the in vivo antitumor effects of MORAB-003 were mediated by late-stage autophagy in cancer cells expressing FRα and that targeting FRα with MORAB-003 is highly effective in reducing tumor growth in orthotopic ovarian cancer models. We conclude that the mechanims of FRα blockade–induced inhibition of tumor growth is autophagy-related cell death. Moreover, these data identify potential biomarkers related to autophagy that could be useful during clinical development of MORAB-003.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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