

APR-246 alone and in combination with a phosphatidylserine-targeting antibody inhibits lung metastasis of human triple-negative breast cancer cells in nude mice

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Background: Approximately 15–20% of all human breast cancers are classified as triple-negative because they lack estrogen and progesterone receptors and Her-2-neu, which are commonly targeted by chemotherapeutic drugs. New treatment strategies are therefore urgently needed to combat triple-negative breast cancers (TNBCs). Almost 80% of the triple-negative tumors express mutant p53 (mtp53), a functionally defective tumor suppressor protein. Whereas wild-type p53 (wtp53) promotes cell-cycle arrest and apoptosis and inhibits vascular endothelial growth factor-dependent angiogenesis, mtp53 fails to regulate these functions, resulting in tumor vascularization, growth, resistance to chemotherapy, and metastasis. Restoration of p53 function is therefore a promising drug-targeted strategy for suppressing TNBC metastasis.

Methods: APR-246 is a small-molecule drug that reactivates mtp53, thereby restoring p53 function. In this study, we sought to determine whether administration of APR-246, either alone or in combination with 2aG4, an antibody that targets phosphatidylserine residues on tumor blood vessels and disrupts tumor vasculature, effectively inhibits stem cell-like characteristics of tumor cells and migration in vitro, and metastasis of human mtp53-expressing TNBC cells to the lungs in mouse models.

Results: APR-246 reduced both the stem cell-like properties and migration of TNBC cells in vitro. In mouse models, administration of either APR-246 or 2aG4 reduced metastasis of TNBC cells to the lungs; a combination of the two diminished lung metastasis to the same extent as either agent alone. Combination treatment significantly reduced the incidence of lung metastasis compared either single agent alone.

Conclusion: Metastasis of human mtp53-expressing TNBC cells to the lungs of nude mice is inhibited by the treatment that combines activation of mtp53 with targeting of phosphatidylserine residues on tumor blood vessels. We contend therefore that our findings strongly support the use of combination treatment involving mtp53 activation and immunotherapy in patients with TNBC.

Keywords: breast cancer, metastasis, blood-vessel targeting agent, p53, APR-246

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Introduction

Approximately 200,000 new cases of breast cancer are detected every year in the United States, and 40,000 American women die of the disease annually.¹ Most deaths occur following the emergence of drug-resistant cancer cells and tumor metastasis.¹ Consequently, more effective treatment strategies to reduce breast cancer-related

mortality are urgently needed. Approximately 15–20% of all breast cancers are classified as triple-negative breast cancers (TNBC) because they do not express estrogen receptor, progesterone receptor, or Her-2-neu.^{2–4} Because such tumors lack these targets, currently used chemotherapeutic protocols are largely ineffective against TNBC, making this cancer virtually untreatable.

However, it has recently been shown that almost 80% of the TNBC tumors express a mutant form of the p53 tumor suppressor protein (mtp53) that is functionally defective.⁵ Wild-type p53 tumor suppressor protein (wtp53) promotes cell-cycle arrest and apoptosis and inhibits vascular endothelial growth factor-dependent angiogenesis, which, if left unchecked, leads to rapid tumor growth, metastasis, and patient death.^{6–12} Most p53 mutations occur in the DNA-binding domain, thereby preventing normal regulation of p53 target genes involved in apoptosis, cell-cycle arrest, and/or angiogenesis.^{13,14} Dysregulation of these processes promotes neovascularization, unconstrained tumor growth, and metastasis, and can lead to the development of resistance to chemotherapeutic drugs.¹⁵ Conversely, wtp53 suppresses both the self-renewal properties of stem cells and the epithelial-to-mesenchymal transition, the latter of which is vital for the initiation of tumor metastasis.^{16–18} We contend therefore that restoration of wtp53 functions in women with p53-defective TNBC may represent a viable alternative therapeutic approach to combat this aggressive type of cancer.

APR-246 is a recently developed small-molecule drug that reactivates mtp53 by covalently modifying the DNA-binding core domain of the mutant protein through alkylation of thiol groups, thereby restoring wild-type conformation and function.^{19,20} Functional studies show that APR-246 is converted to methylene quinuclidinone, which covalently binds to cysteine residues in p53 protein and thereby reactivates mtp53.^{20,21} APR-246-facilitated restoration of wtp53 renews its ability to promote cell-cycle arrest and apoptosis of tumor cells,^{22,23} though the capacity of APR-246 and reactivated mtp53 to control TNBC tumor growth, drug resistance, and metastasis, which are the leading causes of TNBC patient death, is not fully known. In human clinical trials, doses of up to 135 mg/kg of APR-246 have been administered, with doses between 60 and 100 mg/kg being well tolerated and found to be clinically useful against hematologic malignancies and prostate cancer.^{24,25}

In this study, we examined the *in vitro* and *in vivo* effects of APR-246 alone, as well as its effectiveness *in vivo* in combination with 2aG4 (human equivalent

bavituximab; US Food and Drug Administration-approved for clinical trials), a tumor blood-vessel-specific antibody that has been shown to reduce tumor angiogenesis^{26–28} and therefore may be able to inhibit tumor cell metastasis. *In vitro*, APR-246 reduced both TNBC cell mammosphere formation and aldehyde dehydrogenase isoform 1 (ALDH) activity, both of which are characteristics of cancer stem cells, the major cause of the emergence of drug-resistant tumors in individuals with TNBC,^{29,30} and inhibited migration of TNBC cells. These findings suggest that APR-246 has the capacity to destroy cancer stem cells, and could represent an innovative therapy to prevent the emergence of drug-resistant tumors in individuals with TNBC. APR-246 also effectively reduced the metastasis of TNBC cells to the lungs in two different mouse models, without any toxicity to experimental animals. Furthermore, a combination of APR-246 and 2aG4 treatment inhibited incidence of lung metastasis even more effectively than APR-246 or 2aG4 alone.

Materials and methods

Cell lines and culture

All cell culture studies were approved by the University of Missouri Institutional Environmental Health and Safety Board (Columbia, MO, USA). MDA-MB-231 and MDA-MB-435 TNBC cells were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-231-4175 LM2 cells³¹ were obtained from Dr. Joan Massague from Memorial Sloan-Kettering Cancer Center (New York, NY). Cells were grown at 37°C in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 5% CO₂ and harvested for various experiments with 0.05% trypsin-EDTA (ThermoFisher Scientific, Waltham, MA, USA). Prior to treatment in fresh medium containing 5% FBS, cells were washed with phosphate-buffered saline (PBS).

Reagents

APR-246 (PRIMA-1^{MET}) was purchased from Tocris Bioscience (Bristol, UK). For *in vivo* studies, APR-246 was kindly provided by APREA AB (Solinka, Sweden). 2aG4, a mouse IgG2a monoclonal antibody that binds directly to phosphatidylserine on tumor blood vessels,³² was provided by Dr. Rolf Brekken from University of Texas Southwestern Medical Center (Dallas, TX, USA). Binding of 2aG4 to anionic phospholipids relies on a 50-kD bovine plasma

glycoprotein, β 2-glycoprotein 1. Consequently, we mixed 2aG4 at a 1:1 ratio with β 2-glycoprotein 1 to enhance binding of 2aG4 to exposed anionic phospholipids on the endothelial cell surface.³³ The IgG2a mouse anti-colchicine monoclonal antibody C44 (also provided by Dr. Brekken) was used as a negative control for 2aG4.

ALDH activity

TNBC cells were washed once with PBS and harvested using Accutase (BD Biosciences, Franklin Lakes, NJ, USA). ALDH activity was measured using the ALDEFLUOR™ kit (STEMCELL Technologies, Vancouver, BC, Canada) and flow cytometry per the manufacturer's protocol. Treatment with the ALDH inhibitor N,N-diethylaminobenzaldehyde was used as a negative control.

Mammosphere-formation assay

MDA-MB-435 and MDA-MB-231 cells were grown in 100 mm dishes in DMEM/F12 medium supplemented with 10% FBS to 60% confluence. Cells were then washed twice in PBS, harvested and counted. Cells (5×10^3) in 0.1 mL complete Mammocult™ medium (STEMCELL Technologies) were seeded onto ultra-low adherent six-well plates (STEMCELL Technologies) containing 1.9 mL/well of complete Mammocult medium supplemented with APR-246 at the final concentrations indicated. For controls, an equal volume of PBS (vehicle) was added to the medium. Incubations were carried out in triplicate. Every 48 hrs, cells were retreated with additional 1 mL of fresh APR-246 (or vehicle) in complete Mammocult medium. Images of mammospheres were captured by EVOS light microscopy (10x objective) (Waltham, MA, USA) on days 2, 4, and 6 of treatment. Mammospheres $\geq 100 \mu\text{m}$ were quantified.

Cell-migration assay

Viable MDA-MB-231 cells were counted and plated (4×10^4 cells/well) on an Oris Pro Cell Migration Assay 96-well tissue culture-treated plate containing a bead of biocompatible gel (Cat # PROCMA1; Platypus Technologies; Fitchburg, WI, USA). Plates were incubated at 37°C for 1 hr to allow the gel to dissolve, leaving a circular zone of detection. Two hours later, once cells had properly adhered to the plates, they were treated with APR-246 (or PBS vehicle) for 24 or 48 hrs. Images were captured at 0-, 24-, and 48-hr intervals on an EVOS XL Cell Imaging System utilizing a 4x objective. Migration was analyzed by determining the difference in area of the detection zone between pre-migration (0 hr) and

subsequent time points (closure), using the lasso and measurement tools in Adobe Photoshop CS 5.5 (San Jose, CA, USA). Inter-well coefficients of variability given by the manufacturer for the detection zone created by the biocompatible gel were $\leq 12\%$.

Animal studies

All animal studies were approved by the Animal Care and Use Committee at the University of Missouri (Columbia, MO, USA). The study adhered to the guidelines of the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. Female athymic nude (nu/nu) mice, 6 weeks old and 20–22 g, were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and housed in a laminar air-flow cabinet under specific pathogen-free conditions. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the National Institutes of Health.

An initial experiment was performed by injecting 2×10^5 MDA-MB-231-4175 (LM2) metastatic cells intravenously (iv) via tail vein.³¹ Five days later, animals received APR-246 (100 mg/kg) or PBS vehicle by intraperitoneal (ip) injection daily for 7 days, then every other day for an additional 15 treatments. Animal weights were recorded twice weekly throughout the study. At the termination of the experiment on day 42, animals were sacrificed and the lungs harvested and fixed in Bouin's Fixative Solution (Cat # 1120-32; Ricca Chemical Company; Arlington, TX, USA). Superficial lung colonies were then counted using a stereoscopic dissection microscope and the results from two independent researchers averaged, then statistically analyzed using SigmaPlot software, version 12.5 (San Jose, CA, USA).

A subsequent study was performed using well-established metastatic MDA-MB-435 breast cancer cells^{34,35} that were harvested by trypsinization and washed twice with DMEM/F12 medium. Animals were inoculated iv with MDA-MB-435 cells (2×10^6) on day 0 and then randomly assigned to four groups, with 10–11 mice/group. Treatment began on day 5 (see Figure 5A for detailed treatment protocol and schedule). One group of animals received APR-246 alone (100 mg/kg/treatment) by iv tail-vein injection. A second group received 2aG4 (100 μg /mouse/treatment) by ip injection, while a third group was

given both APR-246 and 2aG4 (100 mg/kg+100 µg) by iv and ip injection, respectively. The study lasted for a total of 57 days from initial inoculation of animals with the MDA-MB-435 cells to animal sacrifice. The control group received ip injections of control antibody C44 (100 µg/mouse/treatment). A total of 19 treatments were performed. Animal weights were recorded twice weekly throughout the study. At the termination of the experiment on day 57, animals were sacrificed, lungs harvested, and fixed in Bouin's Fixative Solution (Cat # 1120-32; Ricca Chemical Company; Arlington, TX, USA). Superficial lung colonies were counted and statistically analyzed.

Statistical analysis

Differences among groups were tested using Student's *t*-test or one-way analysis of variance (ANOVA). SigmaPlot software (version 14) was used for statistical analysis. Data are reported as mean±standard error of the mean (SEM). For all comparisons, $P<0.05$ was considered significant. The assumption of the ANOVA was examined, and if necessary, a nonparametric measure based on ranks was used. If normality failed, Kruskal–Wallis one-way ANOVA on ranks was used in place of regular ANOVA. In cases where a significant effect was shown by ANOVA (F-ratio, $P<0.05$), the Student–Newman–Keuls multiple comparison test was employed to compare the means of the individual groups. Differences in the incidence of lung metastasis were examined using logistic analysis of variances performed using the GENMOD procedure in the SAS program (Cary, NC, USA).

Results

APR-246 inhibits ALDH activity in TNBC cells

ALDH activity correlates with the presence of the stem cell phenotype and metastatic potential in TNBC cells.³⁶ With this in mind, we exposed both MDA-MB-231 and MDA-MB-435 TNBC cells to different doses of APR-246 to ascertain its effect on ALDH activity. Treatment of cells with 10–50 µM APR-246 significantly reduced the percentage of ALDH-positive cells by up to 90% compared with controls (Figure 1A and B). A dose of 50 µM APR-246 effectively abolished the incidence of ALDH-positive cells in both cell lines examined.

APR-246 inhibits formation of mammospheres by TNBC cells

Mammosphere-formation assays demonstrate anchorage-independent growth and are an excellent tool for examining

cancer stem cells and progenitor cells.³⁷ We next assessed whether APR-246 could interfere with cancer stem cell enrichment and thereby reduce the formation of mammospheres by TNBC cells. MDA-MB-231 and MDA-MB-435 TNBC cells were subjected to mammosphere-formation assays in the presence of different doses of APR-246 for up to 6 days. In PBS vehicle-treated wells, mammosphere formation increased steadily over the 6-day period (data not shown). However, lower concentration of APR-246 (1 µM) significantly reduced mammosphere formation in both cell lines and higher concentrations of APR-246 (5 or 10 µM) completely eradicated mammosphere formation at all time points in both cell lines examined (Figure 2A and B, data shown for day 4), suggesting that APR-246 effectively diminishes the cancer stem cell pool in TNBC cells.

APR-246 inhibits migration of MDA-MB-231 TNBC cells

We used MDA-MB-231 cells to test whether APR-246 suppresses TNBC cell migration, which is an indicator that cells have acquired the ability to metastasize.³⁸ Compared with controls, APR-246 significantly interrupted migration of MDA-MB-231 cells dose-dependently, with cell migration inhibited more than 50% in response to treatment with 25 µM APR-246 for 24 or 48 hrs (Figure 3).

APR-246 inhibits metastasis of MDA-MB-231-4175 (LM2) TNBC cells to the lungs in nude mice

Using the MDA-MB-231-4175 (LM2) TNBC cell line, which has been selected for lung metastasis,³¹ we examined whether APR-246 could suppress metastasis of TNBC cells to the lungs. Compared with control-treated mice, APR-246 treatment significantly reduced the number of metastatic colonies in the lungs of nude mice (Figure 4, upper panel) with no loss of animal weight (Figure 4, lower panel), indicating that the treatment was not toxic.

APR-246 alone and in combination with antibody 2aG4 inhibits metastasis of MDA-MB-435 TNBC cells to the lungs in nude mice

We used a well-developed mouse model^{34,35} to study the effects of APR-246 when administered either alone or in combination with 2aG4, an antibody that targets tumor vasculature, on metastasis of MDA-MB-435 TNBC cells

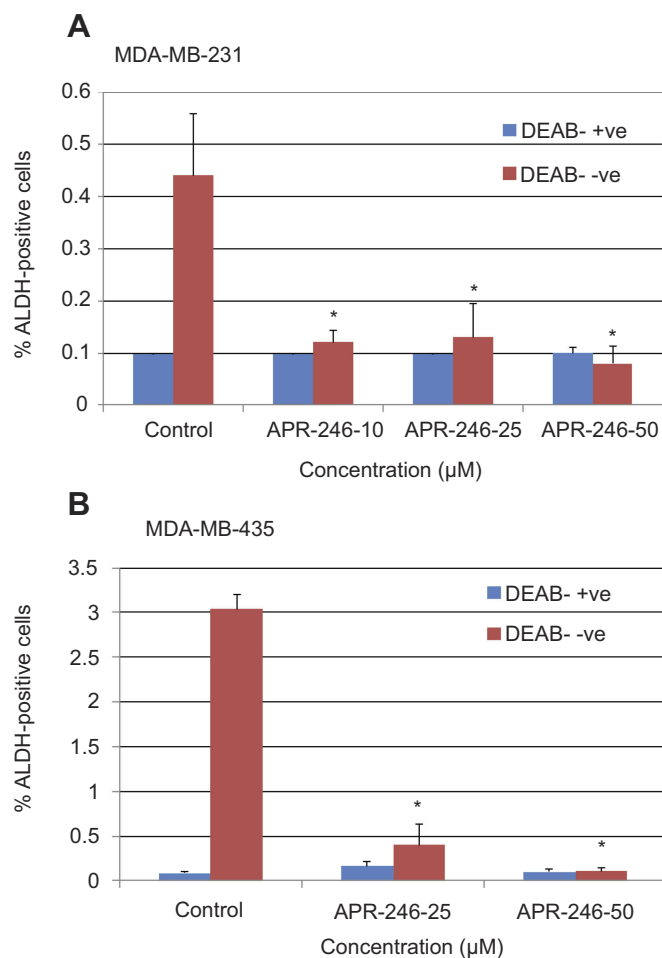


Figure 1 APR-246 inhibits ALDH activity in TNBC cells.

Notes: MDA-MB-231 (**A**) or MDA-MB-435 (**B**) TNBC cells were treated in petri dishes with APR-246 or PBS vehicle (control) DMEM/F12 with 5% FBS for 24 hrs, then incubated with ALDEFLUOR substrate \pm 5 mM DEAB (an inhibitor of ALDH) as described by the ALDEFLUOR assay kit's manufacturer (STEMCELL Technologies). Cells (2.5×10^4 cells/sample) were subsequently analyzed using flow cytometry. The ALDH gate was set using the negative control (+ DEAB). Viable cells were gated using propidium iodide. Results represent mean \pm SEM (n=3). *Significantly different from control ($P < 0.05$, one-way ANOVA) followed by a Student–Newman–Keuls multiple comparison test.

Abbreviations: ALDH, aldehyde dehydrogenase isoform I; ANOVA, analysis of variance; DEAB, N,N-diethylaminobenzaldehyde; PBS, phosphate-buffered saline SEM, standard error of the mean; TNBC, triple-negative breast cancer.

to the lungs (Figure 5A). Animals maintained their weight throughout the study (Figure 5B), showing that the treatments were non-toxic. The experiment was terminated on day 57 and lung tissues collected for photography and analysis. Compared with control-treated animals, those administered APR-246 or 2aG4 alone exhibited significantly fewer metastatic lung colonies (Figure 6A and B). The greatest effect was observed with a combination of the two agents, where the number of metastatic colonies was reduced more than 90% compared with control-treated animals. However, the number of lung colonies observed in mice treated with a

combination of APR-246 and 2aG4 was not significantly different from the number seen with individual APR-246 or 2aG4 treatment alone.

Compared with control-treated mice, administration of APR-246 and 2aG4 alone increased the number of animals exhibiting no metastatic lung colonies (ie, reduced incidence of metastasis), but this did not reach significance. However, the incidence of lung metastasis in animals receiving a combination of APR-246 and 2aG4 was significantly different from control-treated animals, and from the use of 2aG4 alone but not the use of APR-246 alone (Figure 7).

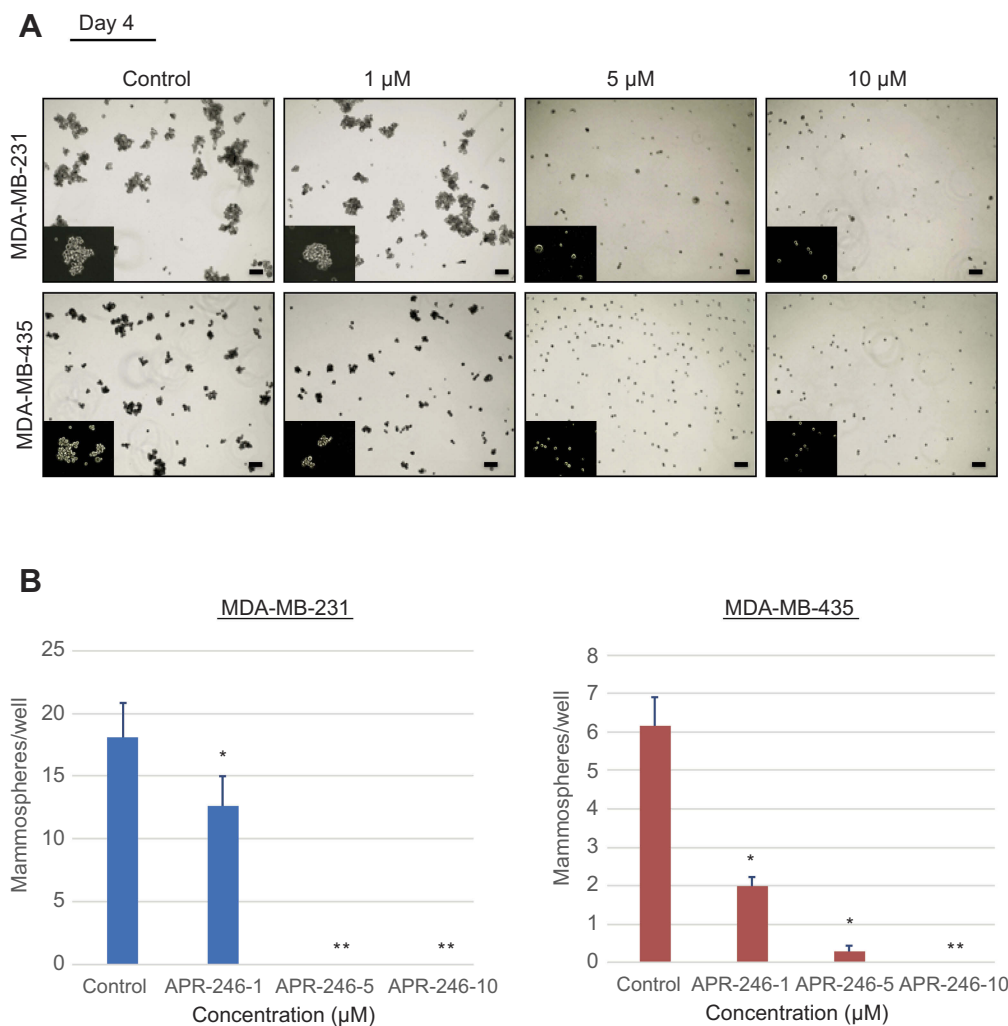


Figure 2 APR-246 inhibits mammosphere formation in TNBC cells.

Notes: MDA-MB-231 and MDA-MB-435 cells were seeded into ultra-low adherent 6-well plates (5×10^3 cells/well) and treated for up to 6 days with 1–10 μM APR-246 or medium (control) in Complete Mammocult medium. Cells were retreated with fresh APR-246 or Complete Mammocult medium every 48 hrs. Representative light microscopic images of mammospheres formed on day 4 are shown in (A) and bar graph representing inhibition of mammospheres is shown in (B). Bar represents 100 μm . Inserts show an enlarged image of mammosphere. *Significantly different from control ($P < 0.05$, one-way ANOVA) followed by a Student–Newman–Keuls multiple comparison test. **Significantly different from control and 1 μM APR-246 groups ($P < 0.05$, one-way ANOVA) followed by a Student–Newman–Keuls multiple comparison test.

Abbreviation: TNBC, triple-negative breast cancer.

Discussion

The presence, within a tumor, of stem-cell-like cells with the potential to metastasize is a major hallmark of metastatic breast cancer.^{39,40} Two important biomarkers of stem-cell-like cells in breast cancer are increased ALDH activity and the ability to form mammospheres.³⁷ Our findings that the mtp53 activator APR-246 interfered with two such important components of metastatic cancer in two well-established mtp53-expressing TNBC cell lines suggest that it has the ability to reduce the population of stem-cell-like cells within a tumor and therefore

inhibit metastasis. Using in vitro assays of MDA-MB-231 TNBC cells, we further found that APR-246 also suppressed cell migration, another hallmark of invasive breast cancer cells that go on to establish metastatic colonies at secondary sites.³⁸

Initial studies in a mouse model of metastatic breast cancer using MDA-MB-231–4175 (LM2) TNBC cells³¹ demonstrated that APR-246 treatment reduced the number of metastatic colonies observed in the lungs of nude mice. Additional in vivo studies in a second mouse model of metastatic breast cancer indicated that

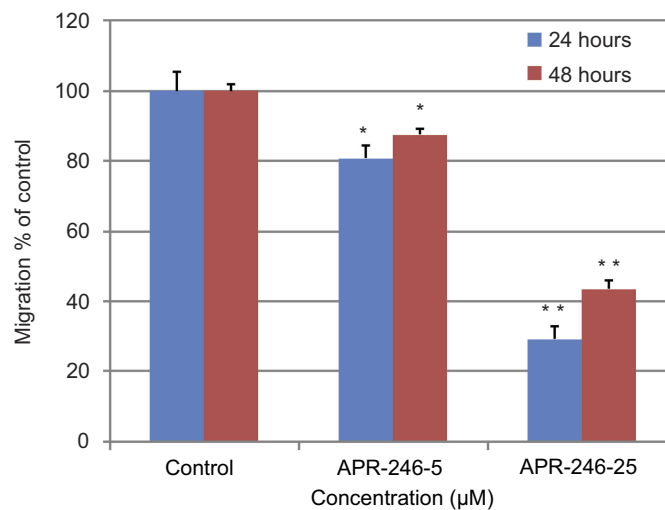


Figure 3 APR-246 inhibits migration of MDA-MB-231 TNBC cells.

Notes: MDA-MB-231 cells (4×10^4 cells/well) were plated in a 96-well migration-assay plate and subjected to cell-migration assays in the presence of APR-246 (5 or 25 μM) or PBS (control) for 24–48 hrs. Pictures were taken just prior to initiation of treatment (0 hr; pre-migration) and after 24 and 48 hrs. Bar graph quantitates the difference in cell migration from pre-migration compared with control (value set at 100%) as described in Methods. Data represent mean±SEM (n=6). *Significantly different from controls; **Significantly different from APR-246-5 ($P < 0.05$, one-way ANOVA) followed by a Student–Newman–Keuls multiple comparison test).

Abbreviations: ANOVA, analysis of variance; PBS, phosphate-buffered saline; SEM, standard error of the mean; TNBC, triple-negative breast cancer.

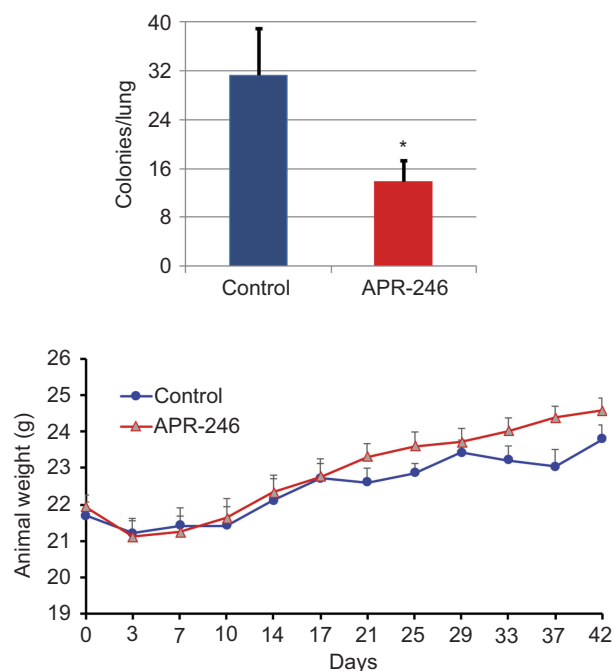


Figure 4 APR-246 inhibits metastasis of MDA-MB-231-4175 (LM2) TNBC cells to the lungs in nude mice.

Notes: MDA-MB-231-4175 (LM2) cells (2×10^5) in 0.1 mL DMEM medium were injected into nude mice via tail vein and allowed to grow and circulate for 5 days. Animals were randomly assigned to two groups and treated with APR-246 (100 mg/kg; ip) or PBS (control) every day for 7 days, then every other day for an additional 15 treatments. Left, lungs were then removed and the number of metastatic colonies determined. Data represent mean±SEM (n=6 animals/group). *Significantly different from controls ($P < 0.05$; Student's *t*-test). Right, animals were weighed every 3–4 days throughout the experiment shown on the left. Data represent mean±SEM (n=6). No significant weight difference was found between the two groups using two-way repeated measures ANOVA (SAS Proc GLM).

Abbreviations: i.p., intraperitoneal; SEM, standard error of the mean; TNBC, triple-negative breast cancer.

administration of APR-246, either alone or in combination with 2aG4, an antibody that targets tumor vasculature, reduced the number of metastatic colonies derived from MDA-MB-435 TNBC cells in the lungs, and that a combination of APR-246 and 2aG4 also reduced incidence of lung metastasis in nude mice. APR-246's ability to interrupt TNBC cell migration could underlie at least in part the reduced number of metastatic colonies in the lungs that we observed with APR-246 treatment in the two different mouse models of metastatic breast cancer employed in this report. However, in previous studies, we showed that APR-246 activated the mtp53 protein and induced apoptosis in hormone-dependent breast cancer cells.^{41,42} Consequently, in the studies presented here APR-246 may inhibit formation of metastatic lung colonies by activating mtp53 or by eradicating mtp53-containing tumor cells in the process of extravasation into the lungs since APR-246 treatment was started 5 days after injection of TNBC cells. These postulates remain to be tested.

Our finding that 2aG4 alone also reduced lung metastasis indicates that the antibody most likely prevented the growth of tumor-specific blood vessels that facilitate expansion of metastatic colonies following microscopic infiltration. However, we cannot rule out important recent observations that 2aG4 may also cause a heightened immune response and subsequent infiltration of immune cells to the site of metastasis in *in vivo* breast cancer metastasis models.^{43,44} This

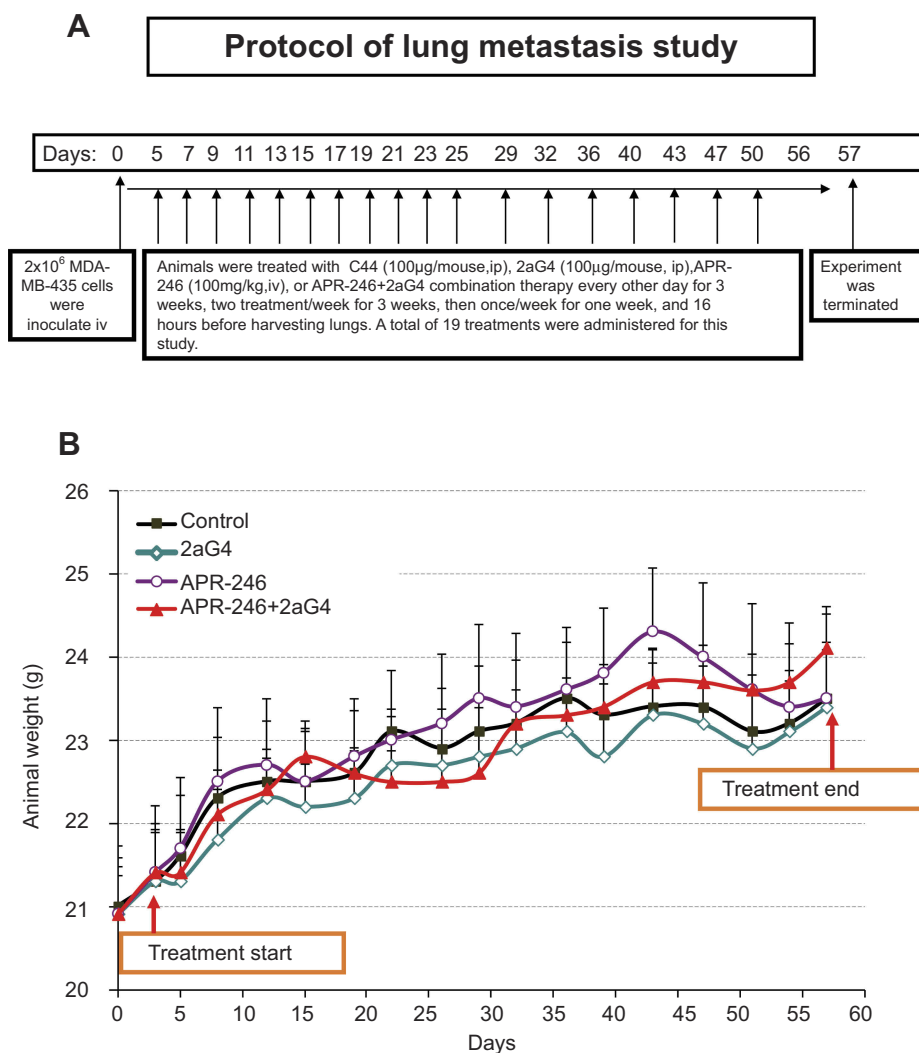


Figure 5 Protocol for lung metastasis study.

Notes: (A) Protocol for examining the effect of APR-246 and 2aG4 antibody alone or in combination on lung metastasis of MDA-MB-435 TNBC cells in nude mice. (B) Animal weight during the treatment protocol shown in (A). Animals were weighed every 3–4 days throughout the experiment. Data represent mean \pm SEM (n=10–11/group). Control animals were treated with C44 antibody (ip). No significant weight differences were observed between control-treated animals and those administered APR-246 or 2aG4 alone or in combination using the one-way ANOVA on ranks (Dunn's test).

Abbreviations: ANOVA, analysis of variance; ip, intraperitoneal; SEM, standard error of the mean; TNBC, triple-negative breast cancer.

response may also contribute to reduced formation of metastatic lung colonies or prevent the cell migration and invasion necessary for the metastatic process, two possibilities that will be the subject of future studies. For example, the influence of 2aG4 on the infiltration of immune cells will be examined in syngeneic tumor models.

Conclusion

In conclusion, our study provides a strong rationale for using a therapy regimen that combines APR-246 and 2aG4 to treat and prevent metastatic breast cancer in lungs originating from mtp53-expressing TNBC cells. The former targets and activates mtp53, likely inducing

apoptosis of metastatic cells, while the latter disrupts tumor blood vessels and may also activate an immune response in tissues. Our in vivo animal studies showed no signs of toxicity with any of the regimens tested, leading us to propose that such therapy could prove beneficial and safe for women with TNBC, who currently have few effective treatment options. Furthermore, because the p53 mutation is common in other types of cancer and angiogenesis is essential for the spread of all types of tumors,^{45,46} we contend that our findings support further study of APR-246 and 2aG4 for treating human TNBC, as well as other forms of cancer.^{47,48}

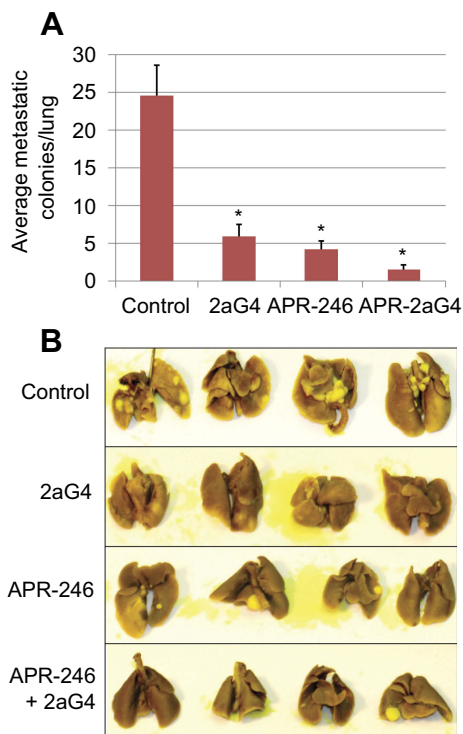


Figure 6 APR-246 and 2aG4 treatment inhibits metastasis of MDA-MB-435 TNBC cells to the lungs in nude mice.

Notes: (A) Female nude mice were inoculated with MDA-MB-435 cells and then treated with C44 antibody (control), APR-246, 2aG4, or a combination of APR-246 and 2aG4 following the protocol described in Figure 5A. Data represent mean number of lung colonies \pm SEM ($n=10-11$ /group) obtained at the end of the experiment. *Significantly different from control ($P<0.05$, ANOVA on ranks followed by Dunn's test). (B) Representative images of lungs from four different animals from each group; colonies appear as off-white specks on the lungs (an example is circled).

Abbreviations: ANOVA, analysis of variance; SEM, standard error of the mean; TNBC, triple-negative breast cancer.

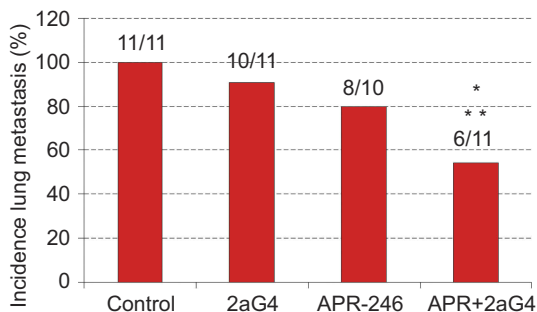


Figure 7 A combination of APR-246 and 2aG4 inhibits incidence of lung metastasis in animals inoculated with MDA-MB-435 TNBC cells.

Notes: Animals ($n=10-11$) were inoculated with MDA-MB-435 cells. After treatment with C44 antibody (control), APR-246, 2aG4, or a combination of APR-246 and 2aG4 following the protocol described in Figure 5A, the number of animals with lung metastasis (incidence) among groups were compared using logistic analysis of variances performed with the GENMOD procedure in the SAS program. *Significantly different from control; **Significantly different from 2aG4 ($P<0.05$).

Abbreviations: GENMOD, generalized linear models theory; SAS, statistical analysis software; TNBC, triple-negative breast cancer.

Abbreviations

ALDH, aldehyde dehydrogenase isoform 1; ANOVA, analysis of variance; FBS, fetal bovine serum; ip, intraperitoneal; iv, intravenous; mtp53, mutant p53 tumor suppressor protein; PBS, phosphate-buffered saline; SEM, standard error of the mean; TNBC, triple-negative breast cancer; wtp53, wild-type p53 tumor suppressor protein.

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Disclosure

SMH is the Zalk Missouri Professor of Tumor Angiogenesis. RAB reports grants from Oncologie, during the conduct of the study and grants from Peregrine Pharmaceuticals, outside the submitted work. In addition, RAB has multiple patents issued related to PS targeting licensed to UT System. The authors report no other conflicts of interest in this work.

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