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Targeting mutant p53 protein and the tumor vasculature: an effective combination therapy for advanced breast tumors

Yayun Liang,

Dalton Cardiovascular Research Center, University of Missouri-Columbia, 134 Research Park Drive, Columbia, MO 65211, USA

Cynthia Besch-Williford,

Department of Pathobiology, University of Missouri, Columbia, MO 65211, USA

Indira Benakanakere,

Dalton Cardiovascular Research Center, University of Missouri-Columbia, 134 Research Park Drive, Columbia, MO 65211, USA

Philip E. Thorpe, and

Department of Pharmacology and Simmons Cancer Center, University of Texas Southwestern Medical Center, Dallas, TX 75235, USA

Salman M. Hyder

Dalton Cardiovascular Research Center, University of Missouri-Columbia, 134 Research Park Drive, Columbia, MO 65211, USA. Biomedical Sciences, University of Missouri, Columbia, MO 65211, USA

Salman M. Hyder: hyders@missouri.edu

Abstract

Breast cancer progression depends upon the elaboration of a vasculature sufficient for the nourishment of the developing tumor. Breast tumor cells frequently contain a mutant form of p53 (mtp53), a protein which promotes their survival. The aim of this study was to determine whether combination therapy targeting mtp53 and anionic phospholipids (AP) on tumor blood vessels might be an effective therapeutic strategy for suppressing advanced breast cancer. We examined the therapeutic effects, singly, or in combination, of p53 reactivation and induction of massive apoptosis (PRIMA-1), which reactivates mtp53 and induces tumor cell apoptosis, and 2aG4, a monoclonal antibody that disrupts tumor vasculature by targeting AP on the surface of tumor endothelial cells and causes antibody-dependent destruction of tumor blood vessels, leading to ischemia and tumor cell death. Xenografts from two tumor cell lines containing mtp53, BT-474 and HCC-1428, were grown in nude mice to provide models of advanced breast tumors. After treatment with PRIMA-1 and/or 2aG4, regressing tumors were analyzed for vascular endothelial growth factor (VEGF) expression, blood vessel loss, and apoptotic markers. Individual drug treatment led to partial suppression of breast cancer progression. In contrast, combined treatment with PRIMA-1 and 2aG4 was extremely effective in suppressing tumor growth in both models and completely eradicated approximately 30% of tumors in the BT-474 model. Importantly, no toxic effects were observed in any treatment group. Mechanistic studies determined that PRIMA-1 reactivated mtp53 and also exposed AP on the surface of tumor cells as determined by enhanced 2aG4 binding. Combination treatment led to significant induction of tumor cell apoptosis, loss of

Correspondence to: Salman M. Hyder, hyders@missouri.edu.

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VEGF expression, as well as destruction of tumor blood vessels. Furthermore, combination treatment severely disrupted tumor blood vessel perfusion in both tumor models. The observed in vitro PRIMA-1-induced exposure of tumor epithelial cell AP might provide a target for 2aG4 and contribute to the increased effectiveness of such combination therapy in vivo. We conclude that the combined targeting of mtp53 and the tumor vasculature is a novel effective strategy for combating advanced breast tumors.

Keywords

Breast tumor growth; Blood vessel targeting agent; p53; p53 Reactivation and induction of massive apoptosis (PRIMA-1); Apoptosis; Cell proliferation

Introduction

As the molecular changes leading to various cancers are being identified, it has become possible to target specific molecules and thereby develop innovative cancer treatment strategies. Currently, two main approaches of molecular targeting are being evaluated in clinical practice, therapeutic monoclonal antibodies (mAbs) and small-molecule agents, both of which show great promise for future therapies based on target proteins [1,2].

Mutation of the p53 tumor suppressor gene is the most common genetic alteration in human cancer. Approximately, 50% of all breast cancers carry point mutations in the p53 gene (mtp53), and the majority of mtp53 alleles in breast cancer cells are defective in DNA binding, cell cycle checkpoints, and DNA damage-induced induction of apoptosis [3,4]. Mutations in p53 or the p53 pathway are thought to play a key role in promoting tumor cell survival and resistance to chemotherapeutic drugs [5,6]. Consequently, restoration of p53 function within tumors leading to apoptosis or cell cycle arrest has been pursued as a promising strategy for cancer therapy [7–10]. One approach is to reactivate endogenous mutant p53 (mtp53) by stabilizing its wild-type conformation. The first reported success of this approach was with the small molecule CP-31398 [11]. Another approach involves re-activating the endogenous mtp53 protein [7,8]. p53 Reactivation and induction of massive apoptosis (PRIMA-1) is a small molecule compound that has the ability to convert mtp53 into an active conformation, thereby restoring the sequence-specific DNA-binding and transcriptional activation function of p53 [12]. This in turn leads to apoptosis, cell-cycle arrest, and hence suppression of human tumor xenografts [12]. We previously reported that PRIMA-1 restores wtp53 sequence-specific DNA-binding and transcriptional activation of p21 in BT-474, HCC-1428, and T47-D cells, which all harbor mtp53 [13]. We also found that PRIMA-1 inhibits hormone-induced vascular endothelial growth factor (VEGF) expression in these breast cancer cells and suppresses BT-474, T47-D, and HCC-1428 breast tumor growth in nude mouse models [14–16].

A tumor-specific vasculature is needed for tumor growth and survival [17,18]. Thus, tumor vasculature has also been considered as a molecular target for antitumor therapy [19]. Vascular targeting agents for the treatment of cancer are designed to rapidly and selectively shut down the tumor vasculature via ischemia and extensive hemorrhagic necrosis [19], resulting in cell death by the indirect action of starving tumor cells of their blood supply. These agents may therefore be effective against tumors that are resistant to conventional antiproliferative chemotherapy [19,20]. Two major types of vascular targeting agents, ligand-based agents and small molecules, are being developed to treat cancer, both of which demonstrate impressive antitumor effects in murine tumor models [19]. Anionic phospholipids (AP) are markers of tumor blood vessels [21], which can be targeted by antibodies to achieve antitumor effects [22]. The 3G4 antibody is a mouse IgG3 monoclonal

antibody that binds specifically to AP on the surface of tumor blood vessels in the presence of β 2-glycoprotein 1 and disrupts tumor vasculature [22–24]. A new isoform of 3G4 and 2aG4 is a mouse IgG2a monoclonal antibody, which has the same binding characteristics [25]. Preclinical studies demonstrate that both 3G4 and 2aG4 have potent antitumor effects in several models [22–25].

Because PRIMA-1 and 2aG4 are effective anticancer agents with unique target mechanisms, we sought to determine whether a combination therapy with the two drugs might additively or synergistically suppress advanced breast cancer. We examined the effects of PRIMA-1 and 2aG4 on xenografts derived from two types of human breast cancer cells, BT-474 and HCC-1428. BT-474 cells express mtp53 and estrogen and progesterone receptors (ER⁺ and PR⁺), as well as abundant levels of HER2-neu [26]. This cell type forms invasive and aggressive breast tumors in vivo that metastasize to the lymph nodes of nude mice [27]. HCC-1428 is a metastatic breast adenocarcinoma cell line that also expresses mtp53 but has a slower growth rate than BT-474 xenografts [16,28]. HCC-1428 cells are also ER⁺ and PR⁺, but HER2/neu negative, and also form invasive breast tumors in nude mice. Our findings from these studies indicate that a combination of PRIMA-1 and 2aG4 is indeed an effective therapy that could provide a novel strategy for suppressing breast cancers, particularly those which have reached an advanced stage.

Materials and methods

Cell lines and culture

The breast cancer cell lines BT-474 and HCC-1428 and human umbilical vein vascular endothelial cells (HUVECs) were obtained from ATCC (Manassas, VA, USA). BT-474 cells were grown in phenol red-free DME/F12 medium (Invitrogen Corporation & Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA). HCC-1428 cells were grown in RPMI-1640 medium (ATCC) supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 g glucose/l, and 1500 mg sodium bicarbonate/l. HUVECs (passages 4–6) were cultured in F-12K medium supplemented with 15% FBS, 0.1 mg/ml heparin, and 0.05 mg/ml endothelial cell growth supplement. All cells were grown in 100 × 20 mm² tissue culture dishes and harvested with 0.05% trypsin–EDTA (Invitrogen).

Animals

Five- to six-week-old female nude (nu/nu) mice weighing 18–22 g were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN, USA). The mice were 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.

Determination of the effects of PRIMA-1 on restoration of wtp53 conformation by immunofluorescence double-staining analysis

BT-474 and HCC-1428 cells were seeded into 8-well chamber slides overnight, washed, and treated with or without 25 and 50 μ M PRIMA-1 (Tocris Bioscience, Ellisville, MO, USA) for 3- and 8-h at 37°C. After two washes with Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen), the cells were fixed with 4% paraformaldehyde for 18 min. The cells were then washed thrice with D-PBS for 5 min per wash and permeabilized with 0.2% Triton X-100 for 3 min. Following three more washes with D-PBS, non-specific binding was blocked with 5% goat serum-PBS for 60 min at room temperature (RT). Cells were washed and incubated overnight with or without PAb240 (1:50 dilution in 5% goat serum in PBS) or

PAb1620 (1:40 dilution in 5% goat serum in PBS) antibodies (Chemicon International Inc., Temecula, CA, USA), after which they were washed thrice with D-PBS and incubated with a rhodamine-conjugated goat anti-mouse IgG antibody (CalBiochem; 1:100 dilution) for 1 h in the dark at RT. Cells were then incubated with DAPI (Invitrogen; 1:5000 dilution) for 10 min to counterstain nuclei, washed six times with D-PBS (5 min/wash), mounted on slides with 90% glycerol in PBS, and coverslipped. The coverslips were sealed with nail polish. Images were captured using a Coolsnap digital camera mounted on an Olympus microscope and processed with Image-Pro Ex (ver. 5.1.0.12, Bethesda, MD, USA).

In vitro detection of exposed AP on the surface of breast cancer cells and HUVECs by immunofluorescence double-staining analysis

2aG4, a mouse IgG2a monoclonal antibody that binds directly to AP on tumor blood vessels, was provided by Peregrine Pharmaceuticals, Inc. (Tustin, CA, USA). Binding of 2aG4 with AP depends on the presence of a 50-kDa bovine plasma glycoprotein, β_2 -glycoprotein 1 (β_2 gp1); we therefore mixed 2aG4 1:1 with β_2 gp1 to enhance binding of 2aG4 with the exposed AP on the surface of endothelial cells [23]. C44, an IgG2a mouse anti-colchicine monoclonal antibody, was used as a negative control for 2aG4. C44 and β_2 gp1 were provided by Dr. Thorpe from University of Texas Southwestern, Dallas, TX, USA. BT-474 (1.5×10^4 /well) and HCC-1428 breast cancer cells (1.0×10^4 /well), or HUVEC (0.7×10^4 /well), were seeded into 8-well chamber slides and grown overnight. To induce phosphatidylserine exposure, cells were treated with either PRIMA-1 at 5, 10, or 25 μ M for 24 h or H_2O_2 (100 or 200 μ M) for 1 h at 37°C. [Higher concentrations of PRIMA-1 induce significant apoptosis of cells and cells cannot be assessed for exposure of AP.] After treatment, the cells were washed and incubated with 2aG4 (2 μ g/ml) for 1 h at RT. They were then washed twice with D-PBS and fixed with 200 μ l 4% paraformaldehyde for 18 min. After three further washes with D-PBS (5 min/each), the cells were permeabilized with 200 μ l 0.1% Triton X-100 for 5 min, washed three more times with D-PBS, and incubated with a rhodamine-conjugated goat anti-mouse antibody (1:100 dilution) mixed with FITC-labeled phalloidin to stain the cytoskeleton (1:40 dilution; Invitrogen) for 1 h in the dark at RT. The cells were washed six times with D-PBS (5 min/wash), mounted on slides with 90% glycerol in PBS, and coverslipped, and the coverslips were sealed with nail polish. Images were captured using a Coolsnap digital camera mounted on an Olympus microscope and processed with MetaVue software (ver. 7.0.4, Molecular Devices, Sunnyvale, CA, USA).

Targeted experimental therapy with PRIMA-1 and 2aG4 alone or in combination in nude mice bearing advanced breast cancer xenografts

Five- to six-week-old nude mice weighing 18–22 g were inoculated with a 17- β -estradiol pellet (1.7 mg/pellet, 60-day release; Innovative Research of America, Sarasota, FL, USA) 48-h prior to injection of tumor cells. BT-474 or HCC-1428 cells were harvested by trypsinization, washed twice with DMEM/F12 medium, and resuspended (5×10^6 cells) in a 0.15-ml solution of DMEM/F12:Matrigel (BD Biosciences, Bedford, MA, USA) (1:4). Cells were injected subcutaneously into both flanks of each mouse, and tumors were measured at 3-day intervals after inoculation. Treatment was started when tumor volumes reached 150–200 mm³ for BT-474 and 250–300 mm³ for HCC-1428 xenografts. Animals were assigned to four groups of either eight mice per group (HCC-1428) or nine mice per group (BT-474). Animals in the BT-474 tumor model group were treated with PRIMA-1 (50 mg/kg/day by intravenous [iv] injection), 2aG4, or C44 (100 μ g/mouse/day), or PRIMA-1 plus 2aG4. Experimental agents were administered three times per week for 4 weeks. Those animals assigned to the HCC-1428 tumor model group were treated with PRIMA-1 (75 mg/kg/day; iv), 2aG4, or C44 (100 μ g/mouse/day), and PRIMA-1 plus 2aG4 every other day for 4 weeks. Tumors were measured every 3 days with a digital caliper and tumor volumes

calculated by the formula $\{(L \times W \times H) \times \pi/6\}$. Animals were weighed at the start of the study and every 3 days thereafter until the end of the experiment when they were killed and tumors harvested. Portions of freshly collected tumor tissue were immediately placed in 4% paraformaldehyde solution for immunohistochemistry or saved in liquid nitrogen for further analysis.

Immunofluorescence double-staining of apoptotic tumor cells

The fragmented DNA of apoptotic tumor cells was detected using a commercial terminal dUTP-mediated nick end labeling (TUNEL) staining kit (Promega Corp., Madison, WI, USA). In brief, frozen sections of tumors from mice treated as described above were washed with PBS and fixed with 4% paraformaldehyde for 10 min. The sections were then permeabilized by incubation with 0.2% Triton X-100 in PBS for 15 min, incubated with equilibration buffer, and drained. A reaction medium containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase was added. The sections were then incubated in a humidified chamber for 1 h at 37°C in the dark. The reaction was terminated by immersing the sections in 2× SSC (30 mmol/l NaCl, 3 mmol/l sodium citrate, pH 7.2) for 15 min, followed by three washes to remove unincorporated fluorescein-dUTP. Sections were subsequently stained with DAPI (0.5 µg/ml) for 5 min, and then mounted with anti-fade mounting media (Vector Laboratories, Inc., Burlingame, CA, USA) and coverslipped prior to examination with UV light. Images were captured using a DP-70 digital camera (Olympus America, Inc., Center Valley, PA, USA) mounted on a Zeiss light microscope and processed with DP Manager software.

Immunofluorescence triple-staining to determine apoptotic endothelial cells

To determine apoptotic endothelial cells, TUNEL labeling with DAPI counterstaining was performed as described above. Prior to DAPI staining, tissues were probed with anti-CD31 antibodies (1:100 dilution of a rat monoclonal antibody [CM 303 A], BioCare Medical, LLC, Concord, CA, USA) to detect endothelial cells. To detect the CD31 signal, a Texas Red-conjugated secondary antibody (TI-9400, Vector Laboratories, Inc.) was used. To evaluate endothelial cell apoptosis, images of the same region of the tumor were captured digitally using 550/590BP filter for Texas Red and 490/520BP filter for FITC fluorescence. To detect DAPI signal a 380/420BP filter was used. When images were merged, apoptotic endothelial cells that were dually labeled were expected to yield a yellow color for detection.

Determination of functional tumor blood vessels by in vivo perfusion assay using FITC-dextran fluorescence

Five- to six-week-old nude mice weighing 18–22 g were inoculated with a 17- β -estradiol pellet (1.7 mg/pellet) 48 h before inoculation with BT-474 or HCC-1428 tumor cells as described in the “Materials and methods” section. When tumor volume reached 150–250 mm³, the animals were treated with PRIMA-1 and/or 2aG4 for 3 weeks. All animals were injected with 0.2 ml of 25 mg/ml FITC-dextran (molecular weight 2,000,000; Sigma-Aldrich, St. Louis, MO, USA) by tail vein 20 min before being killed. Whole blood samples were collected and stored at 4°C in the dark. Blood samples were centrifuged at 15000 rpm for 10 min at 4°C and supernatants collected for fluorescence assay. Tumors were harvested, weighed, and treated with dispase (1:10 dilution, 1 ml per 0.5 g tumor tissue) at 37°C in a shaker for 4 h in the dark. Tumor tissues were then homogenized and centrifuged at 16000 rpm for 15 min. Supernatants were collected and stored in the dark at 4°C. Supernatant fluorescence was measured in a fluorometer (SynergyTM HT, Multi-Detection Microplate Reader, BIO-TEK Instruments, Inc., Winooski, VT, USA). The ratio of tumor fluorescence/plasma fluorescence reflects the extent of tumor blood vessel perfusion.

Routine histochemical and immunohistochemical analysis

Immunohistochemical staining was performed to evaluate expression of VEGF, cleaved-caspase-3, and p21 in tumor cells, and to determine vascularity of tumors by labeling endothelial cells with anti-CD34. Tumor tissue was fixed overnight in 4% paraformaldehyde, followed by paraffin infiltration and embedding. 5 μ m sections were mounted onto ProbeOn Plus microscope slides (Fisher Scientific Inc., Pittsburgh, PA, USA), stained with hematoxylin–Eosin (H&E), and examined for cellularity by light microscopy. For immunohistochemical analysis, unstained paraffin sections were dewaxed in xylene, rehydrated through graded concentrations of ethanol, rinsed in distilled water, and subjected to heat-induced epitope retrieval in 10 mM citrate buffer, pH 6.0 (DAKO, Carpinteria, CA). Slides were treated with 3% H₂O₂ in absolute methanol (to inactivate endogenous peroxidase activity), washed thrice in PBS, and incubated in blocking buffer with 5% bovine serum albumin for 20 min. Sections were treated with the following primary antibodies for 60 min at RT: anti-VEGF (1:100 dilution of a rabbit polyclonal anti-VEGF [sc-152], Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-CD34 (1:25 dilution of a rat monoclonal anti-CD34 [ab8158-100], Abcam, Inc., Cambridge, MA), anti-cleaved caspase-3 (1:100 dilution of a rabbit polyclonal anti-cleaved caspase-3 [2305-PC-100], Trevigan, Gaithersburg, MD); and anti-p21 (1:200 dilution of a rabbit polyclonal anti-p21 [sc-397], Santa Cruz Biotechnology, Inc.). Sections were washed and sequentially incubated with appropriate secondary antibodies. Tissues labeled with antibodies to VEGF, anti-cleaved caspase 3, and p21 were incubated for 30 min with EnVision+, a horseradish peroxidase-labeled polymer conjugated to anti-rabbit antibodies (DAKO). Sections probed with anti-CD34 were incubated for 30 min with a biotinylated rabbit anti-rat IgG (DAKO) and after a wash, with a streptavidin-linked horseradish peroxidase product (DAKO) for another 30 min at RT. Bound antibodies were visualized following incubation with 3,3'-diaminobenzidine solution (0.05% with 0.015% H₂O₂ in PBS; DAKO) or NovaRED substrate (Vector Laboratories, Inc.) for 3–5 min. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared, and coverslipped for microscopic examination.

Quantification of tumor vessels and VEGF immunostaining

VEGF expression was quantified by measuring the immunolabeled pixels in standardized digital images (photographed at 20 $\times$ magnification) of tumor tissue using the Fovea Pro 3.0 imaging program (Fovea Pro 3.0, Reindeer Graphics, Asheville, NC). VEGF distribution was determined on all cells in each tumor image. Eight to twelve images per treatment group from three to five tumors were analyzed. Data are reported as the average number of labeled pixels per group. For blood vessel enumeration, CD34-labeled tissue sections from three to four tumors per treatment group were photographed at 20 $\times$ magnification. From these digital images, total numbers of vessels were counted in 8–15 fields per treatment group (each field represents approximately 0.39 mm²). Vessel density was calculated as vessel number per field and plotted as mean $\pm$ SEM. Data were analyzed using one-way analysis of variance (ANOVA) and $P < 0.05$ was considered significant.

Western blotting for demonstrating the effects of different therapies on expression of Bcl-2 (survival protein)

Whole cell extracts of tumor tissues were prepared using a whole cell extract kit (Active Motif, Carlsbad, CA). In brief, tumor tissue was weighed and diced into small pieces on ice using a sterilized knife. Pieces of tissue were collected in a prechilled centrifuge tube, and the tissues were disrupted and homogenized in ice-cold Complete Lysis buffer using 3 ml/g tissue. Supernatants were transferred into prechilled microcentrifuge tubes and incubated on ice for 30 min, centrifuged thrice at 15000 $\times g$ at 4°C for 20 min, transferred to prechilled microcentrifuge tubes, aliquoted, and stored at –80°C. For western blot analysis, samples containing 50 μ g of protein were separated in a NuPAGE 10% Bis–Tris Gel (Invitrogen).

Electrophoresis was performed at 120 V for 1.5 h using NuPAGE MES-SDS Running Buffer. Separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) at 35 V for 1.5 h. Blots were blocked at RT for 1 h in TBS containing 0.1% Tween 20 (TBS-T) and 5% non-fat dry milk and incubated with Bcl-2 (1:200 dilution) primary antibody (Santa Cruz, CA) for 2 h at RT. Blots were washed thrice with TBS-T and incubated with secondary antibody for 1 h at RT before being washed a further seven times with TBS-T. Immunoreactive bands were visualized using an ECL Plus detection kit (Amersham, Pharmacia Biotech, Arlington Heights, IL). Membranes were stripped and reblotted for β -actin (Sigma), which was used as a control for protein loading.

Statistical analysis

Differences among groups were tested using ANOVA with repeated measures over time. The assumption of the ANOVA was examined, and a non-parametric measure based on ranks was used if needed. Values are reported as mean $\pm$ SEM. When ANOVA indicated a significant effect (F -ratio, $P < 0.05$), the Student–Newman–Keuls multirange test was employed to compare the means of the individual groups. Statistical analyses were performed using SigmaStat software version 3.5.

Results

PRIMA-1 converts mtp53 protein into wtp53 conformation in breast cancer cells

To determine the potential antitumor effect of combination therapy using PRIMA-1 and 2aG4, we first designed in vitro immunofluorescence staining experiments as described in the “Materials and methods” section to confirm that PRIMA-1 converts mtp53 to an active wtp53 conformation. We used two specific antibodies: PAb240, which recognizes only the mtp53 conformation, and PAb1620, which exclusively stains the wtp53 protein [12]. Treatment of BT-474 or HCC-1428 cells with 25- and 50- μ M PRIMA-1 decreased PAb240 staining while concomitantly increasing PAb1620 staining in a time- and dose-dependent manner in both cell lines (Fig. 1a). Most of the stain appeared in the nucleus with either PAb240 or PAb1620, indicating PRIMA-1 most likely converted the p53 conformation within the nucleus. PRIMA-1 (25 and 50 μ M) exerted its maximal effect at 8 h, after which 80–90% of the cells stained positive for the active p53 conformation (data not shown).

PRIMA-1 exposes AP on the surface of breast cancer cells in vitro

AP, which contains the target for the 2aG4 antibody, is expressed on the surface of proliferating tumor endothelial cells [21]. We examined whether PRIMA-1 increases the exposure of AP in the breast cancer cell lines to be tested in this study, both in vitro and in vivo. BT-474 and HCC-1428 cells were seeded into 8-well chamber slides overnight, treated with varying doses of PRIMA-1 (5, 10, and 25 μ M) for 24 h at 37°C, and processed for AP detection as described in the “Materials and methods” section. PRIMA-1 induced AP in both BT-474 and HCC-1428 cells in a dose-dependent manner (Fig. 1b), with staining intensity considerably higher in HCC-1428 cells than in BT-474 cells.

H₂O₂-induced stress exposes AP in HUVEC, a phenomenon that has been demonstrated using both 3G4 [22–24] and 2aG4 [25]. We sought to determine whether PRIMA-1 also exposes AP on the surface of HUVEC cells. We first used H₂O₂ as a positive control, and 100–200 μ M H₂O₂ did significantly induce exposure of AP, as determined by 2aG4 staining (Fig. 1c). However, AP exposure was not significantly enhanced by PRIMA-1 in cultured HUVEC cells compared with controls (Fig. 1c, right two panels). The effects of PRIMA-1 therefore appear to be specific to tumor epithelial cells.

Combined treatment with PRIMA-1 and 2aG4 additively inhibits growth of BT-474 tumor xenografts

We established BT-474 tumor xenografts in nude mice and began treatment with PRIMA-1 or 2aG4 once tumor volumes reached approximately 200 mm³ (Fig. 2a). The tumors continued to grow in presence of the control antibody C44, whereas treatment with 2aG4 inhibited tumor development by 30% and treatment with PRIMA-1 inhibited tumor development by 50% (Fig. 2b, left panel). Importantly, the combination of PRIMA-1 and 2aG4 inhibited tumor growth additively (approximately 75%) over a 4-week treatment (Fig. 2b, bar graphs). The animals did not lose weight during the experiment (Fig. 2b, right bottom panel), indicating that the treatment protocol was not toxic. Thus, the combined administration of PRIMA-1 and 2aG4 was effective at limiting the growth of xenografts derived from BT-474 breast cancer cells.

Combination treatment completely eradicated BT-474-derived tumors in some animals

Some animals with BT-474 xenografts became tumor-free after 4 weeks of combination treatment (Fig. 2c, d), and the combination therapy had a synergistic effect on tumor eradication (Fig. 2d; bar graph). Out of 18 total tumors in each group, 1 (5.6%) was totally eradicated in the 2aG4 group, 2 (11.1%) in the PRIMA-1 group (two different animals), and 5 tumors (27.8%) were eradicated in the group given both PRIMA-1 and 2aG4. The latter group included two animals in which both tumors were eradicated and one in which only one of the two tumors was eradicated, while the size of the second tumor was greatly reduced.

Enhanced antitumor effect of combined PRIMA-1 and 2aG4 treatment in the HCC-1428 tumor model in nude mice

HCC-1428 tumors were allowed to develop in nude mice, and treatment was initiated once tumors were approximately 250–300 mm³ (Fig. 3a). The PRIMA-1 dose was increased to 75 mg/kg/day in the single and combination treatment groups because of the larger initial tumor size compared with BT-474 xenografts, and because the IC₅₀ value for HCC-1428 cells was approximately twofold higher compared with IC₅₀ value for BT-474 cells in the in vitro studies (16). In the control group (C44), tumors continued to develop; however, tumor volumes were reduced by 40.4, 43.5, and 66% following 4 weeks of treatment with 2aG4, PRIMA-1, or a combination of PRIMA-1 and 2aG4, respectively (Fig. 3a–c). However, in contrast to BT-474-generated tumors, none of the HCC-1428-derived tumors were completely eradicated during the time frame tested. As with the BT-474 model, there were no signs of toxicity in any of the treatment groups; body weight, an indicator of the health of the animals, was unchanged at the end of the 4-week study (Fig. 3d).

In vivo perfusion assay of tumor blood vessels

To ascertain whether the treatment protocol used to curtail BT-474 and HCC-1428 tumor growth influenced tumor blood vessel perfusion, we treated tumor xenografts for 3 weeks with PRIMA-1, 2aG4, or a combination of the two agents and then injected 0.2 ml of 25 mg/ml FITC-dextran (molecular weight 2,000,000) iv. After 20 min, animals were killed, whole blood collected, and tumors harvested. After tumors were processed and the homogenate centrifuged as described in the “Materials and methods” section, we measured fluorescence in the supernatant obtained from homogenized tumor tissue and in whole body plasma. All treatments reduced blood perfusion of tumors (Fig. 4a, b) in xenografts from both breast cancer cell types; however, a combination of PRIMA-1 and 2aG4 significantly decreased tumor blood vessel perfusion in both BT-474 (66%) and HCC-1428 (66%) tumor models, compared with single-agent treatment regimens using 2aG4 (51 and 47%) or PRIMA-1 (42

and 44%) alone ($P < 0.05$). Thus, combination treatment with 2aG4 and PRIMA-1 was more effective in blocking tumor blood vessel functions than single agent alone or controls.

Inhibition of VEGF levels and tumor blood vessel density in treated tumors

At the end of the experiments shown in Figs. 2b and 3a, animals were killed. Tumor tissues were collected and immunohistochemically analyzed for expression of VEGF. Expression of CD-34 (a marker for blood vessels) was measured and tumor blood vessel density was determined in excised tumor tissues (Fig. 5a–c). Compared with controls, both VEGF and CD-34 expressions were reduced by each treatment. Tumor blood vessel densities were reduced significantly by combination treatment in xenografts obtained from both breast cancer cell types (Fig. 5c).

Treatment with PRIMA-1 and 2aG4 leads to induction of apoptosis in tumor tissues but not in endothelial cells

A TUNEL assay showed extensive apoptosis in cross sections of xenografts obtained from animals treated with combination therapy (Fig. 6a), and lower (though still substantial) levels of apoptosis following treatment with single agents.

Our earlier studies indicated that PRIMA-1 was ineffective against endothelial cells in vitro [14,16]; however, its effect on endothelial cells in vivo remains unknown. Consequently, using triple fluorescent labeling, we determined whether combination therapy induced apoptosis of endothelial cells (Fig. 6b). We found that while tumor blood vessel density (CD-31 staining, top panel) was reduced with each treatment and was most notable following combination therapy, apoptosis of endothelial cells (detected by presence of yellow color after merger) was absent. This indicates that the reduced tumor blood vessel density occurs as a result of loss of tumor cells and lack of VEGF (Fig. 5a).

Detection of apoptosis-related markers in regressing tumor tissues

In order to further elucidate the mechanisms leading to tumor regression and apoptosis, we measured immunohistochemically the expression of apoptotic markers in BT-474 xenografts from the experiment described in Fig. 2b, and in 1,428 xenografts from the experiment described in Fig. 3a. H&E staining of tissue from both tumor models showed an increase in the number of morphologically apoptotic cells following treatment with either experimental agent (Fig. 6c, top panels for both BT-474 and HCC-1428), confirming results obtained with the TUNEL assay in Fig. 6a, b. Staining of tissue from animals in the combination treatment group showed increased numbers of apoptotic cells compared with control and single treatment groups. We also stained tumor tissues for caspase-3, an indicator of mitochondrial-dependent apoptosis (Fig. 6c, middle panel). Caspase-3 was elevated in regressing tumors indicating that mitochondrial-dependent apoptosis was predominant in regressing tumors (Fig. 6c, middle panels), as also reported by us previously [16]. We also measured expression of p21, another marker for apoptosis/cell cycle arrest, and found that p21 was elevated in treated xenografts (Fig. 6c, bottom panels), confirming that extensive tumor cell apoptosis occurred in xenografts. Using western blot analysis, we found that levels of the survival protein Bcl-2 were reduced in regressing BT-474-derived tumors. In fact, compared with the C44 control group, all treatments reduced Bcl-2 levels; inhibition was 75% (PRIMA-1 + 2aG4), 72% (PRIMA-1 alone), and 55% (2aG4 alone) (Fig. 6d).

Discussion

In this study we show that the dual targeting of breast tumors by reactivation of epithelial cell mtp53 with PRIMA-1, together with disruption of tumor blood vessels by binding 2aG4 antibody with AP exposed on endothelial cells, induces tumor cell apoptosis. This two-

pronged approach severely attenuated the progression of breast disease in our experimental models. Tumors were grown from BT-474 and HCC-1428 cells. In both models combination therapy was more effective than single treatment alone. No toxic effects were observed with any of the treatment regimens used in our study. Importantly, a significant number of BT-474 tumor xenografts were completely eradicated following combination treatment. Since complete tumor eradication did not occur in xenografts derived from HCC-1428 cells, at least not within the timeframe of the study, it may be that more than one mechanism is responsible for the antitumor effects produced by combined therapy with PRIMA-1 and 2aG4.

Using fluorescent staining studies, we demonstrated that in both BT-474 and HCC-1428 cells, PRIMA-1 significantly elevated the level of p53 with the wt conformation. PRIMA-1 also increased TUNEL staining in tumor tissues. Since wtp53 induces the expression of apoptotic genes, and we previously showed that PRIMA-1 leads to increased DNA binding and induction of p21 in cells that express mtp53 [13–15], we propose that reactivated p53 induces apoptotic genes in both BT-474 and HCC-1428 cells. PRIMA-1 is also known to induce apoptotic genes in other breast cancer cell lines, such as MDA-MB-231 cells [29]. In support of such a mechanism, western blot analysis showed that bcl-2 production was significantly inhibited by exposure to PRIMA-1 and 2aG4 in all treatment groups, whereas immunohistochemistry data revealed that expression of p21 was increased in all the three treatment groups compared with controls. Immunohistochemical data also confirmed that in both models combination treatment inhibited VEGF production, a finding consistent with our previous studies in progesterone-dependent and prevention models, where PRIMA-1 was shown to reduce VEGF levels both in vitro and in vivo [14,15]. By virtue of its capacity to reduce production of VEGF, a vital cellular survival factor, PRIMA-1, clearly demonstrates an important antiangiogenic property; this likely promotes the indirect destruction of endothelial cells by reducing the level of both endothelial and epithelial cell-derived VEGF [30,31]. As we have already seen, PRIMA-1 also promotes direct epithelial cell destruction by reactivating p53. Interestingly, triple staining studies showed that none of the treatments induced endothelial cell apoptosis, indicating that loss of blood vessels is likely a result of lack of de novo synthesis rather than destruction of endogenous vessels following treatment with PRIMA-1 or 2aG4. However, since our analysis was conducted following the collection of tumors at the end-point of the experiment, several days after treatment was stopped, we cannot completely rule out loss of blood vessels via destruction of endothelial cells at earlier stages of tumor regression. Such a delay in tumor collection time can also explain why we were unable to see a significant response with respect to loss of VEGF following single treatment in HCC-1428 cells compared with BT-474 cells (Fig. 5b). VEGF levels were approximately fourfold higher in BT-474 cells compared with HCC-1428 cells. In response to various treatments, there was a greater suppression of VEGF signal in BT-474 cells than in HCC-1428 cells. Tumors were collected 5–8 days after the termination of treatments, possibly allowing VEGF to rebound. While the difference in the level of VEGF remained significantly suppressed within treatment groups of BT-474 xenografts, the rebound was most likely sufficient in HCC-1428 cells to remove the statistical significance in the single-agent treatment groups compared with controls. Further studies in which treatment with various agents are given long-term, followed by tumor collection immediately following termination of treatment, and analysis of VEGF will likely shed further light on these possibilities.

Mechanistically, it is possible that the increased antitumor activity resulting from a treatment regimen consisting of both PRIMA-1 and 2aG4 could be due to effects on different signal transduction pathways. PRIMA-1 activates mtp53 and induces the expression of apoptotic genes in tumor epithelial cells, thereby causing cell death via the mitochondrial-dependent apoptotic pathway [14,16]. 2aG4 mediates antibody-dependent cellular cytotoxicity by

binding to cell-surface AP on tumor endothelial cells, effectively shutting down tumor blood vessel function [21,22]. Additionally, since PRIMA-1 promotes apoptosis of tumor epithelial cells, it most likely leads to the generation of reactive oxygen species, which in turn increases local AP exposure on the surface of tumor endothelial cells. This would enhance the ability of 2aG4 to shut down blood vessel function in tumor tissue. Comparable mechanisms have been described for other chemotherapeutic strategies involving a combination of vascular targeting agents [24,25]. Since PRIMA-1 induces AP exposure in tumor epithelial cells, combination therapy with 2aG4 may prove additively effective, since the antibody not only neutralizes tumor epithelial cells, but also disrupts blood vessels. Such a scenario is supported by our perfusion data, which shows that tumor blood vessels and blood vessel perfusion were both reduced. If it could be demonstrated that PRIMA-1 exposes AP in cancer cells that contain wtp53, combination therapy involving PRIMA-1 and 2aG4 may also prove beneficial in such cells, a notion that remains to be determined.

It is interesting to note that BT-474 cells express high levels of Her-2-neu, which is overexpressed in 20–30% of human breast cancers and is associated with a poor prognosis and poor response to hormonal and chemotherapeutic treatment [32–37]. As a consequence, a number of strategies have been proposed for treating Her-2-neu positive tumors [38], though targeting AP exposure has not been thoroughly explored in this regard. Our data provide evidence that treatment with either 2aG4 or PRIMA-1 alone may be effective for treating such tumors. However, since PRIMA-1 not only reactivates mtp53, but also exposes AP sites on tumor epithelial cells, it is likely that combination therapy using both agents would be most effective. It is also interesting to speculate that administration of PRIMA-1, followed in sequence by 2aG4, may prove to be even more effective against tumors that express both Her-2-neu and mtp53, since PRIMA-1 would cause apoptosis of tumor cells and also pave the way for subsequent 2aG4 action by exposing AP. This idea remains to be tested.

In summary, our study supports the strategy of combination therapy which targets mtp53 with PRIMA-1 and tumor blood vessels with 2aG4. We contend that such treatment could be effective against advanced breast tumors, as well as other cancers. More than 50% of breast cancers express mtp53, which serves as a prognostic indicator and also leads to drug resistance. Our data suggest that the combination therapy proposed herein is likely to arrest tumor growth in a significant number of patients.

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Fig. 1.

a PRIMA-1 converts the conformation of mtp53 in breast cancer cells into the wtp53 form. BT-474 and HCC-1428 cells were grown in 8-well chamber slides and treated with 25- μ M (PM25) or 50- μ M (PM50) PRIMA-1 for 3 and 8 h. Conformation-specific antibodies and imaging procedures were used as described in the “Materials and methods” section. Nuclei are stained with DAPI (*blue*), p53 is stained *red*, and in the merged images, nuclear p53 is *pink*. **b** PRIMA-1 exposes AP on the surface of breast cancer cells in vitro. Breast cancer cells were grown in 8-well chamber slides and treated with 5, 10, or 25 μ M PRIMA-1 for 24 h at 37°C. Exposure of AP was examined by live-cell immunofluorescence double-staining as described in the “Materials and methods” section. Externalized AP were labeled with 2aG4 and are shown in *red* (rhodamine), the cytoskeleton is in *green* (FITC), and the overlap in the merged images ranges from *yellow* to *orange* based on the intensity captured. **c** Weak exposure of AP induced by PRIMA-1 on HUVEC cells. HUVEC were treated with PRIMA-1 as in (b)

Fig. 2.

a Treatment protocol for human breast tumor xenografts in nude mice. **b** Additive effect of PRIMA-1 and 2aG4 on inhibition of BT-474 breast tumor growth in nude mice. Mice ($n = 9$ per group) were inoculated with 5×10^6 BT-474 cells in both flanks. Tumors were measured every 3 days with a digital caliper to determine volume. (Left) Growth curves for tumors with points representing mean tumor volumes $\pm$ SEM. The combination therapy was significantly more effective than either single treatment alone. $*P < 0.05$ compared with the C44 group, $**P < 0.05$ compared with the C44 and 2aG4 groups. (Top right) Tumor weights at the end of the experiment confirming tumor volumes. (Bottom right) Animal weight following treatment. **c** Tumor images in situ representing loss of tumor growth in nude mice following treatment. Images show tumor size before and after 4 weeks of treatment. **d** Eradication of BT-474 tumors in nude mice following PRIMA-1 + 2aG4 treatment. Images in situ show that some tumors were eradicated from the original tumor sites. Numbers of tumor-free animals after 4 weeks of treatment is shown in the bar graph

Fig. 3.

a–d Enhanced inhibition of HCC-1428 breast tumor growth by PRIMA-1 plus 2aG4 combination therapy. Nude mice ($n = 8$ per group) were injected with HCC-1,428 cells, and tumor growth was measured as described in the “Materials and methods” section. The treatment protocol was identical to that described in Fig. 2a except the PRIMA-1 dose was increased to 75 mg/kg/day. **a** Tumor growth curve. *Points* represent mean tumor volumes $\pm$ SEM in each group of mice; **b** *bar graph* representing tumor weight from the experiment shown in (**a**). **c** Representative in situ tumor image from each group at the end of the experiment. *Circles* represent tumor locations. **d** Graph of animal weight. Values represent mean $\pm$ SEM; $*P < 0.05$ compared with the C44 group, $**P < 0.05$ compared with the C44, PRIMA-1 and 2aG4 groups

Fig. 4.

a, b Tumor blood vessel perfusion assay using FITC-dextran in vivo. Following 3 weeks of treatment with PRIMA-1 and 2aG4, tumor-bearing animals were injected (iv) with 0.2 ml of 25 mg/ml FITC-dextran (molecular weight 2,000,000) for 20 min. Tumors and whole blood were collected, processed, and centrifuged. Fluorescence was measured in supernatant from tumor tissue and whole body plasma. The ratio of tumor/plasma fluorescence reflects the extent of tumor perfusion. Samples were analyzed from four animals per group. *Points* indicate mean values in each group of mice, *bars* indicate SEM. * $P < 0.05$ compared with the C44 group, ** $P < 0.05$ compared with the C44, PRIMA-1, and 2aG4 groups

Fig. 5.

a–c Immunohistochemical analysis of tumor tissue for angiogenesis markers. **a** At the termination of the experiment, tumor tissue sections were stained for VEGF and CD-34 (biomarkers for angiogenesis-related genes). Results demonstrated a significant reduction of both molecules following combination therapy. **b** VEGF expression was quantified using the Fovea Pro 3.0 imaging program (Fovea Pro 3.0, Reindeer Graphics, Asheville, NC). Eight to twelve images from three to five tumors per treatment group were analyzed for VEGF. **c** CD-34-based vessel count. Blood vessels were counted in 8–15 individual images from three to four tumors per treatment group as described in the “Materials and methods” section

Fig. 6.

a Induction of tumor cell apoptosis in xenografts by PRIMA-1, 2aG4, or the combination. Animals with BT-474 xenografts were treated with PRIMA-1 and 2aG4 alone or in combination for 4 weeks; the last treatment was administered 16 h prior to kill and tumor collection. Frozen tissue sections were processed for fluorescence double-staining as described in the “Materials and methods” section. *Green* TUNEL staining, *Blue* nuclear staining of tumor cells with DAPI. *Merged colors* show intense apoptosis in xenograft tissue in the presence of PRIMA-1 and 2aG4. **b** Induction of apoptosis in tumor endothelial cells. Animals with BT-474 xenografts were treated with PRIMA-1 and 2aG4 alone or in combination for 3 weeks. Frozen tissue sections were processed as described in (a). *Red* endothelial cell staining, *Green* TUNEL staining, *Blue* nuclear staining of tumor cells with DAPI. *Merged colors* do not show apoptosis in endothelial cells under any treatment. **c** Immunohistochemical staining of apoptotic markers (caspase-3 and p21) in tumor tissues. H&E staining of sections from both tumor models showed an increase in morphologically apoptotic cells in all the three treatment groups, but apoptotic cells were especially abundant following combination treatment (*upper panels*). Tumor tissues from experiment described in (a) were collected and embedded in paraffin. Staining was performed as described in the “Materials and methods” section. **d** Western blot analysis to assess Bcl-2 levels in tumors. Mice with BT-474 tumors were treated with PRIMA-1 and 2aG4 alone or in combination for 4 weeks, the last treatment occurring 16 h prior to kill. Tumor tissues were harvested, homogenized, and whole cell extracts were prepared. Protein (50 μ g per group) was loaded onto NuPAGE 10% *Bis-Tris* Gel. The *bar graph* shows densitometric analysis of Bcl-2 levels normalized to β -actin internal controls. Results from two independent experiments are shown. *Bar graph* also shows densitometric analysis of Bcl-2 levels normalized to β -actin intensity ($n = 3$)