Doxorubicin Synergizes with 34.5ENVE to Enhance Antitumor Efficacy against Metastatic Ovarian Cancer

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Abstract

Purpose: Novel therapeutic regimens are needed to improve dismal outcomes associated with late-stage ovarian cancer. Oncolytic viruses are currently being tested in patients with ovarian cancer. Here, we tested the therapeutic efficacy of combining doxorubicin with 34.5ENVE, an oncolytic herpes simplex virus transcriptionally driven by a modified stem cell–specific nestin promoter, and encoding for antiangiogenic Vasculostatin-120 (VStat120) for use against progressive ovarian cancer.

Experimental Design: Antitumor efficacy of 34.5ENVE was assessed in ovarian cancer cell lines, mouse ascites–derived tumor cells, and primary patient ascites–derived tumor cells by standard MTT assay. The ability of conditioned medium derived from 34.5ENVE-infected ovarian cancer cells to inhibit endothelial cell migration was measured by a Transwell chamber assay. Scope of cytotoxic interactions between 34.5ENVE and doxorubicin were evaluated using Chou–Talalay synergy analysis. Viral replication, herpes simplex virus receptor expression, and apoptosis were evaluated. Efficacy of oncolytic viral therapy in combination with doxorubicin was evaluated in vivo in the murine xenograft model of human ovarian cancer.

Results: Treatment with 34.5ENVE reduced cell viability of ovarian cancer cell lines, and mouse ascites–derived and primary patient ascites–derived ovarian tumor cells. Conditioned media from tumor cells infected with 34.5ENVE reduced endothelial cell migration. When combined with doxorubicin, 34.5ENVE killed synergistically with a significant increase in caspase-3/7 activation, and an increase in sub-G1 population of cells. The combination of doxorubicin and 34.5ENVE significantly prolonged survival in nude mice bearing intraperitoneal ovarian cancer tumors.

Conclusions: This study indicates significant antitumor efficacy of 34.5ENVE alone, and in combination with doxorubicin against disseminated peritoneal ovarian cancer. Clin Cancer Res; 20(24); 6479–94. ©2014 AACR.

Introduction

Epithelial ovarian cancer is the fifth most deadly cancer in women, with more than 22,000 new cases and 14,000 deaths in the United States in 2013. Two thirds of women present with progressive disease wherein the cancer has already disseminated to abdominal organs or distant sites (1). The 5-year relative survival rate for these patients is less than 30% (1). Primary treatment for ovarian cancer involves cytoreductive surgery and a platinum and/or taxane chemotherapeutic regimen (2). Unfortunately, standard therapies have shown limited efficacy with nearly 70% of patients recurring with chemoresistant disease. Ovarian cancer recurrence is often attributed to a small subpopulation of cancer stem-like cells that maintain or rapidly develop resistance to chemotherapy (3). These stem-like cancer cells are critical for reinitiation of tumor growth in preclinical models, and are capable of serial propagation of the original tumor phenotype in animals (4, 5). Tumor cells isolated from malignant ascites have been described to be more stem-like with higher nestin expression (6, 7). Thus, a treatment regimen designed to preferentially target nestin-expressing cancer cells may have improved therapeutic efficacy and prolonged survival.

The process of angiogenesis is also a key component in enabling ovarian cancer to grow and metastasize (8, 9).
High levels of intratumoral VEGF and VEGF receptor correlate with poor patient prognosis and survival (10), and its increased expression contributes to the formation of malignant ascites, a major burden of disease (11, 12). Inhibitors of the VEGF pathway, such as bevacizumab and VEGF Trap, have increased expression contributes to the formation of malignant ascites, a major burden of disease (11, 12). Inhibitors of the VEGF pathway, such as bevacizumab and VEGF Trap, have

**Materials and Methods**

**Cell lines and reagents**

PA-1, OV-4, OVCAR-3, SKOV3 human ovarian carcinoma cells, and Vero (African green monkey kidney cells) were obtained from the ATCC. Cisplatin-resistant (A2780-CR) and cisplatin-sensitive (A2780-CS) human ovarian cancer cells were a kind gift from Dr. Rajagopalan Sridhar (Department of Radiation Oncology, Howard University Medical School, Washington, DC). SKOV3.ip1-firefly Luciferase were a kind gift from Dr. Kah-Wey Peng (Mayo Clinic, Rochester, MN; ref. 20). PA-1 and OV-4 were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin (100 U/mL and 100 μg/mL) at 37°C and 5% CO2. OVCAR-3, SKOV3, and SKOV3-ip1-fluc were maintained in α-Minimal Essential Medium (αMEM) supplemented with 20% FBS and penicillin/streptomycin (100 U/mL and 100 μg/mL) at 37°C and 5% CO2. Human dermal microvessel endothelial cells (HDMEC) were obtained from ScienCell, and maintained at low passage number in Endothelial Basal Media with supplements (ScienCell) and penicillin/streptomycin (100 U/mL and 100 μg/mL) at 37°C and 5% CO2. Chinese hamster ovary (CHO) cells negative for expression of HSV-1 entry receptors (CHO-K1) and CHO-K1 cells transduced to express human hNectin-1 (CHO-N1) or nectin-2 (CHO-N2) were kindly gifts from Patricia G. Spear (Northwestern University, Chicago, IL; refs. 21, 22). CHO-K1 cells stably expressing human HVEM (herpes virus entry mediator; CHO/A) were as described previously (23). CHO cells were cultured in F12 medium containing 10% FBS, penicillin/streptomycin (100 U/mL and 100 μg/mL) and G418 at 37°C and 5% CO2. Doxorubicin was obtained from Sigma-Aldrich, and dissolved in DMSO, and then further diluted as described in PBS for use.

**Viruses**

Construction of 34.5ENVE has been previously described (17). Briefly, to generate 34.5ENVE, an expression cassette encoding Vstat120 gene regulated by the viral IE4/5 promoter with ICP34.5 regulated by a nestin enhancer–driven promoter was inserted into fHSVQ backbone using HSVQuik technology (24). Revertant ENVE was created by removing the expression cassette containing the ICP34.5 and Vstat120 gene by recombination. Viruses were propagated in Vero cells. Three days after infection, Vero cells and media were harvested and subjected to three cycles of freezing in liquid nitrogen and thawing at 37°C to liberate virions. Cell debris was cleared by centrifugation (400 × g, 20 minutes at 4°C). Virus was pelleted from resultant supernatant by centrifugation at 13,000 × g for 1 hour. Virus titer was determined via plaque-forming assay in Vero cells, with plaque-forming units (PFU) assessed 3 days after infection (24).

**Cell viability assay and Chou–Talalay analysis**

Cancer cells were seeded into a 96-well plate (in 2% FBS media in 100 μl volume), and were treated with vehicle or doxorubicin for 18 hours followed by drug wash-out, and then virus infection. Three days after infection, cell viability...
was measured using a standard MTT assay (Roche Applied Sciences). For synergy analysis, dose–response curves and 50% effective dose (ED$_{50}$) values were determined for each individual treatment (drug or virus). Fixed ratios of the ED$_{50}$ value of drug and/or virus were then used to treat the cells, either individually (as controls) or in combination. CompuSyn software (CombioSyn, Inc.) program algorithm assessed the combination index (CI). Combined dose–response curves were fitted to Chou–Talalay lines, which assessed the combination index (CI). Combined dose–response curves were fitted to Chou–Talalay lines, which assessed the combination index (CI). Combined dose–response curves were fitted to Chou–Talalay lines, which assessed the combination index (CI). CI $< 1$ indicates synergistic interaction, whereas CI $> 1$ is antagonistic, and CI $= 1$ is additive (25–28).

**RT-PCR**

To measure relative nestin expression, RNA was purified from cell pellets (QIagen RNeasy, Qiagen), per the manufacturer’s instructions. cDNA was generated (SuperScript Reverse Transcriptase, Life Technologies), and nestin expression was measured via quantitative RT-PCR (Mastercycler ep realplex, Eppendorf) using SYBR Green (Applied Biosystems). GAPDH was used as an internal control. Primer sequences: GAPDH, forward, 5'-GGAGTCAACCG-GATTITGTCG-3'; reverse, 5'-GGAATCAITATTGGAACATG-TAAACC-3'. Nestin, forward, 5'-TCCAGGAACGGAAAAT-CAAG-3; reverse, 5'-GCCTCTCTAGTCCCTACCTC-3'. To evaluate murine cell contamination of mouse ascites–tumor cells, mouse and human $\beta$-actin was probed. Primer sequences: mouse $\beta$-actin, forward, 5'-GCCCTCTCAGTGGACGAGT-3'; reverse, 5'-GCAACTGTTAGCGTGAGT-3'. Human $\beta$-actin, forward, 5'-CAGACTG- TGCCCATCTATGAGG-3; reverse, 5'-GCCCTCTCAGTGGACGAGT-3'.

**Generation of concentrated conditioned media**

To generate concentrated conditioned media, ovarian cancer cells were infected with the indicated oncolytic viruses (OV) at a multiplicity of infection (MOI) of 2 (two infectious viral particles per cell). One hour after infection, unbound virus was washed away, and serum-free media was added. Twelve to 14 hours after infection, conditioned media was harvested, treated with 0.4% human IgG to neutralize contaminating virus, and centrifuged for 1 hour at 13,000 rpm to pellet any virus in the media before being concentrated 100-fold using 50,000 kDa Amicon Ultra centrifugal concentration filters (Millipore).

**In vitro endothelial cell migration assays**

Endothelial cell migration assays were performed using a modified Boyden chamber assay similar to previous reports (17, 18). Transwell membranes (8 micron Corning Costar) were precoated on the underside with 5 $\mu$g/mL fibronectin (Millipore) in PBS. Migration of serum-starved HDMEC toward conditioned medium (CM) was measured using Transwell chambers. HDMEC were plated in the upper chamber, and cells were allowed to migrate for 12 hours, at which point membranes were fixed in 1% glutaraldehyde and stained with 0.5% crystal violet; unemigrated cells were removed from top chamber (using cotton swab). Images of the membranes were obtained at $\times 20$ magnification, and quantified by counting five fields of view per well ($n = 3$ /group).

**Viral replication assay and viral burst assay**

Cells were treated with doxorubicin for 18 hours at indicated concentrations. Drug was then washed out, and cells were infected at an MOI of 0.001 (for replication assay) or MOI of 1 (for burst assay) in 2% FBS media. Infection proceeded for 1 hour, and then unbound virus was washed away and fresh 2% FBS media was added to each well. Cells were harvested at indicated time points after infection, and titers were determined by standard plaque assay on Vero cells (24).

**Immunoblots**

Immunoblots were probed with rabbit anti–N-terminal BAI1 (29) to probe for VStat120, mouse anti-human GAPDH (Abcam), or mouse anti-ICP4 (Abcam) antibodies, followed by goat anti-rabbit (Dako), or sheep anti-mouse (Amersham Biosciences) secondary antibodies.

**Assay of caspase-3/7 activity**

Cells were seeded onto 96-well plates (in 2% FBS media in 100 $\mu$L volume), and were treated with drug, virus, or combination at indicated concentrations or MOI. Caspase-3/7 activity per $\mu$g protein was evaluated using the Caspase-Glo 3/7 Assay Kit (Promega) and BCA Protein Assay kit (Thermo Scientific) according to the manufacturer’s instruction. For caspase inhibition experiments, 15 $\mu$mol/L of the caspase inhibitor ZVAD–FMK (Promega) was added in tandem with each treatment.

**Animal experiments**

All animal experiments were performed in accordance with the Subcommittee on Research Animal Care at The Ohio State University guidelines, and have been approved by the Institutional Review Board. Six- to 8-week-old female athymic nu/nu mice (18–25 g; Charles River Laboratories) were injected i.p. with $2 \times 10^6$ SKOV3.ip1-fluc cells in 500 $\mu$L sterile saline. All treatment injections with doxorubicin, virus, or mock were administered i.p. in 100 $\mu$L volume. Mice were sacrificed upon significant weight gain due to ascites accumulation, development of significant jaundice or wasting, or development of necrotic subcutaneous lesion at the injection site.

**Cancer cell isolation from murine ascites**

To isolate cancer cells from mice, ascites were collected via intraperitoneal draining using 20G needle from tumor bearing mice with obvious progressive peritoneal disease. Upon collection, ascites volume was increased to 30 mL.
using PBS, and then centrifuged to pellet all cells. Cell pellet was resuspended in 5 mL of ammonium chloride red blood cell lysis buffer (StemCell Technologies); lysis proceeded at room temperature for 5 minutes with rocking. Remaining tumor cells and contaminating immune cells were plated in 20% FBS αMEM, and incubated for 2 to 3 days at 37°C in 5% CO₂. Samples were confirmed for lack of contaminating murine cells using RT-PCR probing for murine-specific β-actin, and then used for experiments as indicated.

**Patient ascites–derived primary ovarian carcinoma cell sample isolation**

Primary ovarian carcinoma cell samples used in this study were isolated from human ascites collected at the time of debulking surgery from patients with high-grade serous ovarian carcinoma; (see Supplementary Table S1 for details about each specimen, including patient age, histologic subtype, and cancer stage grouping). All patients signed consent forms, and the use of patient samples was approved under the Ohio State University Human Investigations Committee (IRB # 0040124). Routine histopathologic examination of formalin-fixed paraffin-embedded tissue from each patient (carried out at the hospital) was followed, and the ascites from confirmed predominant high-grade serous histologic subtype were used for further experiments. Equal volume of patient ascites and warmed RPMI with 20% FBS were plated in T75 flasks (Corning) and incubated at 37°C, with 5% CO₂, and 95% humidified air (30). Fresh medium was replaced 3 to 5 days after initial plating, and every 4 to 5 days until the cells approached confluence. The cells were frozen in RPMI with 10% FBS and 10% glycerol in liquid nitrogen for future use. Characterization for the confirmation of the presence of tumor cells was done using antibodies for pan-cytokeratin (Abcam), vimentin (Abcam), and CD14 (Abcam), with DAPI counterstain (Vector Laboratories). Pan-cytokeratin+/CD14− and Vimentin+ cells have been previously characterized as ovarian cancer cells; coexpression of vimentin and pan-cytokeratin is frequently observed in ovarian cancer cells (30–33). Cultures with >95% pan-cytokeratin+ and vimentin+ and CD14− were used for future experiments. All the experiments were carried out using early passage cells (34, 35).

**Flow-cytometry analysis of HSV receptor expression**

SKOV3 cells were treated with 40 nmol/L doxorubicin for 18 hours before harvesting. Anti-human nectin-1 (R1.302.12, sc-6918; Santa Cruz Biotechnology), nectin-2 (R2.525, sc-32804; Santa Cruz Biotechnology), HVEM (CW10, sc-21718; Santa Cruz Biotechnology), and FITC-conjugated goat anti-mouse IgG (BD Biosciences) were used for HSV entry receptor staining.

**Luciferase imaging**

Luciferase imaging was performed using Xenogen (IVIS) in vivo imaging system (PerkinElmer). Luciferin was used at in vivo concentration as indicated by the manufacturer’s instructions (PerkinElmer).

**Statistical analysis**

For normally distributed measurements (or data transformed to normal), a two-sample t test was used to compare the means of two independent groups. ANOVA models or linear models were used to compare the mean of multiple groups, or to include two or more factors. Two-way ANOVA models with the interaction term were used to evaluate the synergistic effect between two treatments (e.g., doxorubicin and 34.5ENVE). For survival data, survival rates were estimated by the Kaplan–Meier method, and the survival curves were displayed and compared among groups by the log-rank test. All tests were two-sided. P values were adjusted for multiple comparisons using the Holm procedure. An adjusted P value of 0.05 or less was considered statistically significant.

**Results**

**Ovarian cancer cells are sensitive to killing by 34.5ENVE OV, and mediate increased 34.5ENVE replication**

The genetic structures of the oncolytic viral vectors used in this study have been previously described (17, 24, 36), and are outlined in Fig. 1A. Briefly, 34.5ENVE virus is an oHSV-1 with a disrupted ICP6 gene and deletions of both native copies of the ICP34.5 gene. It then has one copy of ICP34.5 driven by a modified nestin promoter reintroduced into the backbone, and also expresses antiangiogenic VStat120 driven by an immediate early viral promoter (called IE4/5; ref. 17). A rescue virus, called revertant ENVE, was generated by removal of the inserted nestin-driven ICP34.5 and IE4/5-driven VStat120 expression cassette from the 34.5ENVE backbone. To determine whether ovarian cancer cell lines were sensitive to 34.5ENVE and revertant ENVE, the indicated cells were infected with virus at increasing MOI, and cell viability was measured by standard MTT assay. Figure 1B shows sensitivity of ovarian cancer cell lines to killing by both 34.5ENVE and revertant ENVE. The two-way ANOVA test indicated that in four of six cell lines tested there is an overall significant difference between sensitivity to killing by revertant ENVE and 34.5ENVE (Fig. 1B). The two cell lines, A2780CR and A2780CS, which only showed a trend toward overall significant difference, were also extremely sensitive to virus killing relative to the other four cell lines, even with the 34.5-deleted revertant ENVE. Ovarian cancer cell lines were also conducive to viral replication, with 34.5ENVE replicating better than revertant ENVE. Figure 1C shows viral replication after 72 hours in several cell lines as measured by standard plaque assay (Student t test; *, P < 0.05; **, P < 0.001; and ***, P < 0.0001; ref. 24).

**Mouse ascites– and patient ascites–derived tumor cells express increased nestin expression and sensitivity to 34.5ENVE**

To evaluate whether increased sensitivity to OV correlated with nestin expression, we compared sensitivity of SKOV3 cells grown in vitro to SKOV3 cells harvested from murine ascites, which develop in mice after tumor implantation. Tumor cells isolated from malignant ascites have been described to be more stem cell–like with higher nestin...
Figure 1. Efficacy of 34.5ENVE against ovarian cancer cell lines in vitro. A, schematic of genetic alterations in the genomes of the OVs used in the study, compared with wild-type parental F strain HSV-1. TR, terminal repeats; IR, internal repeats; U, unique region; L, long; S, short; VStat120, Vasculostatin-120. B, cytotoxicity assay of panel of ovarian cancer cell lines infected with 34.5ENVE (solid line, ●) or revertant ENVE (dotted line, ○) at increasing MOI. Viability was assessed 3 days after treatment using MTT assay. Data, the percentage of viable cells relative to untreated control; error bars, SD. Linear models were used to compare the means of two groups. P values, the overall test result by linear models for the comparison between 34.5ENVE and revertant ENVE across all MOI levels. C, replication assay comparing 34.5ENVE and revertant ENVE in a panel of ovarian cancer cell lines. Cells were infected at a low MOI, and cell lysates and media were harvested after 3 days. Viral titer was determined by standard plaque assay. Data, PFU/mL; error bars, SD (Student t test: *, P < 0.05; **, P < 0.001; ***, P < 0.0001).
Figure A: Relative nestin expression (normalized to SKOV3) for Ascites-1, Ascites-2, and Ascites-3.

Figure B: % Viability vs MOI for Ascites-1, Ascites-2, and Ascites-3. Significance levels are indicated.

Figure C: Relative nestin expression for 140, 163, 179, and 195.

Figure D: Box plot showing relative nestin expression for Normal, Tumor-I/II, and Tumor-III/IV.

Figure E: % Viability vs MOI for 140, 163, 179, and 195. Significant differences are marked.
expression (6, 7). Consistent with published reports, ex vivo SKOV3 cells harvested from ascites from 3 individual mice (Fig. 2A and B, "ascites-1," "ascites-2," and "ascites-3") had more than a 10-fold increase in nestin expression compared with SKOV3 cells grown in vitro as determined by RT-PCR (Fig. 2A). Along with increased nestin expression, the ex vivo ascites-derived tumor cells were significantly more sensitive overall killing by 34.5ENVE compared with revertant ENVE (Fig. 2B; linear models were used to compare the means of two groups).

Although SKOV3, A2780, and OVCAR3 cells were originally isolated from patients with ovarian cancer, and are identified as such according to the ATCC, molecular profiling of commonly used ovarian cancer cells revealed some differences between the molecular profile of ovarian cancer cell lines, and that of most high-grade serous ovarian cancer tissue derived from patients (37). Thus, we analyzed sensitivity of primary ovarian cancer patient ascites-derived tumor cells to 34.5ENVE or revertant ENVE. Ascites from patients with ovarian cancer (deidentified patient numbers 140, 163, 179, 195; described in Supplementary Table S1) were isolated and cultured as described in Materials and Methods section. The ascites-derived tumor cell culture displayed cobblestone monolayer morphology as described previously (Supplementary Fig. S1; ref. 38). The characterization of the cultures was carried out using immunocytochemistry and immunofluorescence using a panel of antibodies to detect expression of pan-cytokeratin, vimentin, and CD14 (Supplementary Fig. S1). Pan-cytokeratin+/CD14+ cells have been previously characterized as ovarian cancer cells (30).

RT-PCR for nestin gene expression revealed that these ascites-derived tumor cells had increased nestin expression 10- to 100-fold as compared with normal ovarian tissue obtained from tissue qPCR array (from patients with endometriosis, carcinoma of the bladder, or leiomyoma of the myometrium, n = 6; Fig. 2C). TissueScan qPCR Array Ovarian Cancer Disease Panel III (Origene) was used to assess nestin expression in 48 human samples. Analysis of primary patient cDNA samples indicated that nestin expression increases with tumor grade (Fig. 2D; ANOVA models used to compare the mean of multiple groups; P value adjusted by the Holm procedure). Consistent with the increased nestin expression, the primary patient ascites-derived tumor cells also had increased overall sensitivity to 34.5ENVE-mediated killing compared with revertant ENVE (Fig. 2E; linear models used to compare the means of two groups).

**Antiangiogenic protein VStat120 is produced by 34.5ENVE after infection of ovarian cancer, and reduces endothelial cell migration in vitro**

We examined the ability of ovarian cancer cells infected with 34.5ENVE to produce and secrete VStat120 by Western blot analysis. Figure 3A (middle) shows presence of Vasculostatin-120 in cell lysates from a panel of ovarian cancer cell lines infected with 34.5ENVE (indicated with arrow). Furthermore, 34.5ENVE infection yielded a more robust production of viral immediate early protein ICP4 (Fig. 3A, top). The presence of secreted VStat120 was also confirmed by Western blot analysis of concentrated conditioned media (CM) derived from SKOV3 and patient ascites–derived cells infected with 34.5ENVE, revertant ENVE, or mock (Fig. 3B).

To assess the functionality of secreted VStat120 produced by infected ovarian cancer cells, we compared the effect of CM from SKOV3 or primary patient ascites infected with the indicated virus, or PBS on endothelial cells migration in a modified Boyden chamber. Briefly, CM from 34.5ENVE-, revertant ENVE-, or mock-infected cells was added to the bottom chamber, and migration of HDMEC toward the bottom chamber was quantified. There was a significant reduction in endothelial cell migration upon treating with VStat120-containing CM from either infected SKOV3 (26.5% reduction, P = 0.0204) or patient ascites (29.6% reduction, P = 0.014; Fig. 3C; ANOVA models used to compare the mean of multiple groups; P value adjusted by the Holm procedure).

**Therapeutic efficacy of 34.5ENVE against disseminated peritoneal ovarian cancer in vivo**

To test therapeutic efficacy of 34.5ENVE against ovarian cancer in vivo, mice with established intraperitoneal SKOV3 tumors were treated with PBS, revertant ENVE, or 34.5ENVE (5 × 10³ PFU) three times, on 8, 16, and 23 days after tumor cell implantation (Fig. 4A; virus treatment indicated with arrows). Kaplan–Meier survival analysis...
revealed a significant increase in median survival time of mice treated with OV as compared with PBS, with a greater prolonging of survival in mice treated with 34.5ENVE as compared with revertant ENVE-treated animals (Fig. 4A; as compared among groups by log-rank test). Median survival was 63 days for 34.5ENVE-treated mice, 49 days for revertant ENVE-treated mice, and 37 days for PBS-treated mice (Fig. 4A; \( P < 0.001 \)).

Luciferase expression by SKOV3 cells permitted live imaging of mice to monitor disease progression using an IVIS. There was a significant reduction in luciferase activity [as indicated by relative light units (RLU) in photons/s] in the 34.5ENVE-treated cohort 28 days after tumor implantation, as compared with PBS (Fig. 4B, luminescent images; Fig. 4C, quantification of luminescent output, \( P = 0.0342 \) between PBS and 34.5ENVE; ANOVA models used to compare the mean of multiple groups; \( P \) value adjusted by the Holm procedure). Interestingly, all mice showed a delayed development of a subcutaneous tumor at the site of tumor injection. Because virus treatment was administered via i.p. injection on the opposite side of the abdomen, this subcutaneous nodule served as an untreated control tumor in each mouse. Eight of 8 mice treated with PBS developed aggressive ascites by the time of sacrifice, whereas only 4 of 8 mice treated with revertant ENVE developed ascites (Fig. 4D). Only 2 of the 8 mice treated with 34.5ENVE showed the development of ascites at time of death, whereas the other 6 mice were sacrificed because of the growth of the untreated subcutaneous tumor. Gross histology of the ascites-free 34.5ENVE-treated mice (6/8) revealed that they were free of disseminated intraperitoneal metastatic tumor seeding (Fig. 4D; tumor nodules indicated with white asterisks).
Doxorubicin synergizes with 34.5ENVE to kill ovarian cancer cells in vitro and in vivo

Briefly, SKOV3 cells were treated with a fixed concentration of doxorubicin (100 nmol/L) and with varying doses of 34.5ENVE administered 18 hours before doxorubicin, at the same time, or 18 hours after doxorubicin. Viability was measured 3 days after infection by MTT assay. Impact of
Figure 5. Synergistic killing of ovarian cancer cells treated with doxorubicin and 34.5ENVE in vitro and in vivo. A, doxorubicin treatment followed by 34.5ENVE is established as dosing regimen. Cytotoxicity assay of SKOV3 infected with 34.5ENVE alone (dotted line, *) at increasing MOI, or in combination with doxorubicin (solid line, ~) using different treatment schedules. (Continued on the following page.)
doxorubicin and 34.5ENVE on cytotoxicity is depicted in Fig. 5A. Treatment with doxorubicin before 34.5ENVE (far right) showed an increase in cell killing compared with 34.5ENVE alone, thus all subsequent experiments were performed with this treatment schedule. Linear models were used to compare the means of two groups; $P < 0.001$ comparing 34.5ENVE treatment with 34.5ENVE with doxorubicin.

We then measured sensitivity of doxorubicin-treated ovarian cancer cell lines to 34.5ENVE. Cells were treated with doxorubicin for 18 hours followed by drug wash-out, and infected with 34.5ENVE for 72 hours. Viability was measured by MTT assay. Reduction in viability by either agent alone was quantified (D1, reduction in cell viability by doxorubicin; D2, reduction in cell viability by 34.5ENVE). Reduction in viability by combination of doxorubicin and 34.5ENVE treatment was also quantified (called D3). Additive effect was calculated as $[D1+D2]$. Synergistic effect was calculated as $[D3-(D1+D2)]$. In all six ovarian cancer cell lines, treatment of cells with both 34.5ENVE and doxorubicin resulted in more-than-additive cell killing (Fig. 5B; two-way ANOVA models with the interaction term were used to evaluate the synergistic effect between two treatments). Because this suggested synergistic cell killing, we next examined the ability of doxorubicin to synergize with 34.5ENVE using the Chou–Talalay synergy analysis method as described previously (25, 26, 28, 39). This analysis is frequently used to investigate synergy between anticancer killing of agents (39–41). Briefly, the ED50 value of doxorubicin and 34.5ENVE were each defined as the dosage yielding 50% cell viability, as compared with untreated controls. To evaluate whether the combination resulted in synergistic cell killing, the cells were treated with doxorubicin, followed by drug wash-out, and then treated with 34.5ENVE. Concentrations of doxorubicin and 34.5ENVE were serially diluted at fixed ratios of 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 times their ED50. Cell viability was measured 72 hours after virus infection. The viability data were used to calculate the CI via the Compusyn program. CI $< 1$ indicates synergistic interaction, whereas CI $> 1$ is antagonistic, and CI $= 1$ is additive. Doxorubicin and 34.5ENVE combination therapy killed the ovarian cancer cells synergistically in all cell lines at most fractions affected (Fa), as indicated by the CI (Fig. 5C). In a parallel experiment, we tested sensitivity of primary patient ascites-derived tumor cells to doxorubicin alone, and in combination with 34.5ENVE. Consistent with the cell line data, synergistic cell killing was observed between doxorubicin and 34.5ENVE in primary patient ascites-derived cell samples (Fig. 5D).

Next, we tested the in vivo relevance of this synergistic cell killing of doxorubicin in combination with 34.5ENVE. Mice with established intraperitoneal tumors were treated with a single dose of doxorubicin (10 μg/g body weight) 8 days after tumor cell implantation. One day later, mice were treated with a single injection of 34.5ENVE ($5 \times 10^7$ PFU). Median survival was 58 days for doxorubicin with 34.5ENVE treatment, 47 days for 34.5ENVE alone, 47 days for doxorubicin alone, and 32.5 days for PBS treated (Fig. 5E). The Kaplan–Meier survival analysis revealed a significant increase in survival of mice treated with combination therapy of doxorubicin with 34.5ENVE as compared with either agent alone (Fig. 5E; survival curves compared among groups by log-rank test; $P < 0.001$).

Combination therapy with doxorubicin and 34.5ENVE does not alter viral infection or replication, but increases cell death and caspase-3/7 activation. Doxorubicin and 34.5ENVE combination treatment synergistically kills ovarian cancer according to Chou–Talalay synergy analysis and in vivo results. To test whether doxorubicin affected virus replication or infection, we first measured its impact on viral transduction over time by measuring increase in GFP-positive–infected cells by FACS analysis. Figure 6A shows no increase in the relative percentage of GFP-positive cells at indicated time points after treatment with increasing concentrations of doxorubicin. Consistent

(Continued.) Briefly, cells were treated with a fixed concentration of doxorubicin (100 nM/L) and with varying doses of 34.5ENVE administered 18 hours before doxorubicin, at the same time, or 18 hours after doxorubicin. MOI was calculated on the basis of number of cells plated at t = 0 hours. Viability was assessed 3 days after treatment using MTT assay. Data, the percentage of viable cells relative to untreated cells; error bars, SD. Linear models were used to compare the means of two groups; $P < 0.001$; P values, the overall test result by linear models for the comparison between 34.5ENVE and 34.5ENVE with doxorubicin across all MOI levels. B, combination therapy with doxorubicin and 34.5ENVE significantly increased cell death, as compared with either therapeutic alone. Briefly, the indicated cells were treated with sublethal doses of doxorubicin for 18 hours, followed by treatment with sublethal doses of 34.5ENVE. Cell viability was measured 3 days after treatment with 34.5ENVE via MTT. Data, the percentage of reduction in cell viability relative to untreated controls; error bars, SD. Reduction in viability by either agent alone was quantified (called D1 for doxorubicin and D2 for 34.5ENVE). Reduction in viability by combination of doxorubicin and 34.5ENVE treatment was quantified (called D3). Additive effect was calculated as $(D1+D2)$. Synergistic effect was calculated as $[D3-(D1+D2)]$. In all six ovarian cancer cell lines, treatment of cells with both 34.5ENVE and doxorubicin resulted in more-than-additive cell killing. Two-way ANOVA models with the interaction term were used to evaluate the synergistic effect between two treatments (e.g., doxorubicin and 34.5ENVE; $P < 0.001$). Chou–Talalay combination interaction analysis of ovarian cancer cell lines (C) and primary patient ascites–derived cells (D) treated with doxorubicin and 34.5ENVE. Briefly, the ED50 value of doxorubicin and 34.5ENVE were each defined as the dosage yielding 50% cell viability at 72 hours following treatment. To evaluate whether the combination resulted in synergistic cell killing, cells were treated with doxorubicin followed by drug wash-out, and then infected with 34.5ENVE at serially diluted concentrations in a constant ratio. Cell viability was measured 72 hours after infection, and these data were used to establish a CI using the Compusyn program. Data, in C and D are CI values at various fractions affected (Fa) for indicated cells. A CI $< 1$, synergistic interaction; whereas CI $> 1$, antagonistic; and CI $= 1$, additive (dotted line). E, the Kaplan–Meier survival curve of doxorubicin, 34.5ENVE, or combination treatment in the murine model of ovarian cancer. Mice inoculated with intraperitoneal SKOV3 cells were treated with one injection of doxorubicin (10 μg/g body weight) 8 days after tumor cell implantation, followed by treatment with a single injection of 34.5ENVE ($5 \times 10^7$ PFU/mouse) 1 day later. Data, Kaplan–Meier survival curves of the different treatment groups. Median survival of PBS-treated mice was 32.5 days, doxorubicin treated was 47 days, 34.5ENVE treated was 47 days, and combination therapy of doxorubicin and 34.5ENVE extended median survival to 58 days (survival curves compared among groups by log-rank test; $P < 0.001$).
Figure 6. Combination therapy with 34.5ENVE and doxorubicin increased apoptosis without altering virus replication in ovarian cancer cells. A, doxorubicin does not affect virus entry into cells as evaluated by measuring GFP-positive SKOV3 using flow cytometry. Cells were pretreated with doxorubicin for 18 hours, followed by wash-out. Cells were then infected with 34.5ENVE at an MOI of 0.001 for 2 hours, followed by wash-out of unbound virions. 8, 24, and 48 hours after infection cells were harvested and fixed, and the percentage of GFP-positive–infected cells was assessed by FACS. Data, the percentage of GFP-positive cells at each indicated time point from two replicates per treatment (black *, 8 hours after infection; light gray ■, 24 hours after infection; dark gray ▲, 48 hours after infection.) B, doxorubicin treatment does not alter the HSV-1 receptor profile on SKOV3 cells. SKOV3 were harvested 18 hours after treatment with or without 40 nmol/L doxorubicin, and HSV-1 receptor expression was evaluated using antibodies against nectin-1, nectin-2, HVEM. Flow-cytometry analysis indicated no change in expression of receptors in doxorubicin-treated sample as compared with untreated control. CHO cells expressing indicated HSV-1 receptors served as positive staining controls (CHO-N1, nectin-1; CHO-N2, nectin-2; and CHO-A19, HVEM). C, virus replication assay indicates that doxorubicin does not increase viral replication. SKOV3 cells were treated with doxorubicin at indicated concentrations. Cells were then infected with 34.5ENVE at a low MOI = 0.001 for 2 hours, followed by wash-out of unbound virions. Cell lysate was harvested at indicated time points, and PFUs were quantified on Vero cells. Data, PFU per mL from each of two replicates (black *, 8 hours after infection; light gray ■, 24 hours after infection; dark gray ▲, 48 hours after infection.) D, virus burst assay indicates that doxorubicin does not increase the number of viral particles produced in a single replication cycle. SKOV3 cells were treated with doxorubicin at indicated concentrations. Cells were then infected with 34.5ENVE at a high MOI = 1 for 2 hours, followed by wash-out of unbound virions. Cell lysate was harvested 24 hours after infection, and PFUs were quantified on Vero cells. Data, PFU per µg protein from two replicates. E, cell-cycle analysis of cells treated with doxorubicin, 34.5ENVE or combination. Briefly, cells were treated with doxorubicin for 18 hours, followed by drug wash-out. (Continued on the following page.)
with this observation, there was no change in HSV-1 receptor expression (nectin-1, nectin-2, HVEM) in SKOV3 cells treated with 40 nmol/L doxorubicin for 18 hours (Fig. 6B). These results suggest that the increased cell killing was not due to increase in virus transduction after treatment of cells with doxorubicin. Furthermore, replication assays and viral burst assays in the presence or absence of doxorubicin showed no change in viral replication over time with increasing concentrations of doxorubicin treatment (Fig. 6C, replication assay; Fig. 6D, viral burst assay). These results suggest that the increased cell killing was not due to increase in virus replication after treatment of cells with doxorubicin.

Doxorubicin is known to induce apoptosis in ovarian cancer cells. To evaluate whether virus treatment increased apoptosis in ovarian cancer cells, we first measured DNA content via flow cytometry to examine changes in the sub-G1 apoptotic population of cells after a 24-hour treatment with single agent or combination therapy. Treatment with both doxorubicin and 34.5ENVE increased sub-G1 apoptotic population of cells, as compared with either therapy alone (Fig. 6E, representative images). We then measured relative caspase-3/7 activation in cells treated with doxorubicin alone or in combination with 34.5ENVE after 24 hours (Fig. 6F, normalized to untreated control; ANOVA models used to compare the mean of multiple groups; P value adjusted by the Holm procedure). Combination therapy of doxorubicin (160 nmol/L) and 34.5ENVE (MOI = 0.04) significantly increased caspase-3/7 activation in SKOV3 cells over either individual therapy, indicating that the synergistic killing may be mediated via increased apoptosis. Pretreatment of SKOV3 cells with the pan-caspase inhibitor ZVAD–FMK (15 μmol/L) effectively blocks caspase activation (Fig. 6G). Figure 6G shows RLU values indicating caspase-3/7 activation in cells treated with doxorubicin alone or in combination with 34.5ENVE with or without ZVAD–FMK. Data, mean RLU per μg protein from treated G0 peak. Top left, untreated (“−”). Top right, doxorubicin alone (“Dox,” 40 nmol/L). Bottom left, 34.5ENVE alone (“34.5ENVE,” MOI = 0.02). Bottom right, doxorubicin with 34.5ENVE (“Combo”). F, caspase-3/7 activity is increased in SKOV3 treated with a combination of doxorubicin and 34.5ENVE, compared with single-agent treatment. Briefly, SKOV3 were treated 160 nmol/L doxorubicin for 18 hours followed by wash-out, followed by 34.5ENVE at an MOI of 0.04 for 24 hours. Caspase-3/7 activity is analyzed by luciferase activity (RLU). Data, mean RLU per μg protein from treated cells with doxorubicin, 34.5ENVE, or combination (ANOVA models used to compare the mean of multiple groups; P value adjusted by the Holm procedure). G, treatment with the pan-caspase inhibitor ZVAD–FMK blocks caspase-3/7 activation in SKOV3 cells treated with doxorubicin and 34.5ENVE. Cells treated with doxorubicin with or without virus were analyzed for caspase-3/7 activity in the presence or absence of ZVAD–FMK. Data, mean RLU per μg protein from treated cells with doxorubicin, 34.5ENVE, or combination (ANOVA models used to compare the mean of multiple groups; P value adjusted by the Holm procedure; “*,” P < 0.001 between dox without ZVAD–FMK and dox + ZVAD–FMK; “#,” P < 0.0001 between 34.5ENVE without ZVAD–FMK and 34.5ENVE + ZVAD–FMK; “*,” P < 0.0001 between combination without ZVAD–FMK and combination + ZVAD–FMK) H, Chou–Talalay analysis of interaction between doxorubicin and 34.5ENVE in SKOV3 in the presence or absence of the caspase inhibitor ZVAD–FMK. Data, CI values at various fractions affected for indicated treatments. A CI < 1, synergistic interaction; whereas CI > 1, antagonistic, and CI = 1, additive.

**Discussion**

Several different oHSV-1–derived viruses, including HSV1716 and fusogenic Synco-2D, have been shown to be effective against metastatic ovarian cancer in preclinical studies (42–44). Patients with late-stage or recurrent ovarian cancer are currently being recruited for clinical trials to evaluate the use of oncolytic measles virus, or Reovirus with or without paclitaxel (NCT00408590 and NCT01199263). Here, we evaluated therapeutic efficacy of 34.5ENVE against disseminated intraperitoneal ovarian cancer in mice. All oncolytic HSV tested in patients to date have been deleted for both copies of the viral ICP34.5 gene. ICP34.5 plays a crucial role in modulating the cellular PKR-driven antiviral immune response (45). It also inhibits the induction of cellular autophagy, another antiviral defense response (46); and is involved in recruiting proliferating cell nuclear antigen to sites of virus replication (47). HSV-1 vectors deleted for ICP34.5 are safe for intracerebral administration, but the resulting virus is severely attenuated for replication (48–50). 34.5ENVE is designed to reintroduce the ICP34.5 gene under the regulation of a modified nestin promoter resulting in an OV with increased potency in nestin-positive cells (17). Nestin is a marker of stem cells, and its expression has been associated with cancer stem-like cells in several malignancies, including glioma, ovarian, and pancreatic cancers (4, 5, 51–53). Here, we tested the sensitivity of primary patient and experimental mouse ascites–derived tumor cells to 34.5ENVE. We also compared the nestin expression of tumor cells derived from ascites that developed in the experimental mouse model of progressive ovarian cancer with the parent cell line grown in vitro. Consistent with previous reports describing the presence of cancer stem-like cells in ascites, we found elevated nestin expression in ascites-derived tumor cells (4, 5). These cells were significantly more sensitive to killing by 34.5ENVE than the parent cell line. In addition, primary patient ascites–derived tumor cells displayed high levels of nestin gene expression, and were sensitive to 34.5ENVE-mediated oncolysis. This is the first study, to our knowledge, to evaluate the use of oHSV-1 against tumor cells derived from freshly isolated malignant ovarian cancer ascites. In addition to expressing a nestin-driven ICP34.5, 34.5ENVE also encodes for Vsteam20, a potent antiangiogenic fragment derived from the extracellular fragment Brain Angiogenesis Inhibitor 1 (BAIL). In our study, we observed reduced endothelial cell migration in vitro, and a decrease in tumor dissemination–associated ascites in vivo. The antiangiogenic properties of Vsteam20 have been attributed
to five thrombospordin type 1 domains contained within it (19). The use of thrombospordin-1 (TSP1) mimetics for ovarian cancer has shown promising results in preclinical models, and treatment with TSP1 mimetics such as ABT-510 reduced tumor growth, ascites fluid, and intraperitoneal dissemination, in an orthotopic, syngeneic model of ovarian cancer (54, 55). However, clinical testing in patients with advanced soft tissue sarcoma, renal cell carcinoma, and melanoma showed disappointing efficacy results with use of ABT-510 as a single agent, and suggested testing it in combination with other agents in future trials (56, 57). Interestingly, 34.5ENVE-treated animals showed a reduction in the formation of ascites along with a reduction in intraperitoneal seeding of tumor cells. The direct cytolytic effects of the virus along with the continuous release of antiangiogenic Vstat120 from infected cells provide 34.5ENVE with a two pronged therapeutic approach not achievable by repeatedly administration of an antiangiogenic peptide.

Here, we tested the impact of combining 34.5ENVE with doxorubicin. Combination of OHSV-1 with DNA-damaging agents has been shown to improve viral replication and antitumor efficacy in several different cancer models (25, 50, 58, 59). Here, we found that doxorubicin and 34.5ENVE killed ovarian cancer cell lines and patient ascites-derived tumor cells synergistically without affecting virus entry or replication. Combination therapy increased sub-G1 population of apoptotic cells more than either therapy alone. We also found that combination therapy significantly increased caspase-3/7 activation and apoptosis. Blockade of effector caspases ablated the synergistic interaction between doxorubicin and 34.5ENVE, suggesting that the combination effect was mediated by caspase activation and apoptosis. This is consistent with previous reports wherein chemotherapy treatment was shown to select for chemotherapy-resistant cancer stem cells, which can then become susceptible to a nestin-driven OHSV-1 vector (52, 60). Doxorubicin-based therapies are frequently prescribed for patients with ovarian cancer who have developed resistance to taxane- and platinum-containing agents. The finding that 34.5ENVE synergizes with this agent underscores the potential significance of testing OHSV therapy in conjunction with doxorubicin for patients with ovarian cancer.

Interestingly, in a previous report, doxorubicin in combination with HSV1716 (an oncolytic HSV1-derived virus deleted for ICP34.5) was found to have an additive effect in non-small cell lung cancer (61). Consistent with these results, we found that combining doxorubicin and 34.5ENVE at the same time yielded additive results. However, altering our treatment schedule yielded different results, indicating that scheduling optimization will be critical for evaluating therapeutic combinations, including OV therapy. Furthermore, mutations in OV backbones have a critical impact on how cellular signaling is altered in different cell types. This highlights the importance of testing efficacy of individual OVs alone and in combination with chemotherapy in preclinical models before testing in patients. TSP1 mimetic ABT-510 has been shown to induce apoptosis in ovarian cancer cells, and it is interesting to speculate whether the apoptotic effect of 34.5ENVE on ovarian cancer cells depends on Vstat120 expression.

In conclusion, we report that doxorubicin-based chemotherapy, a standard-of-care for recurrent or refractive ovarian cancer, can be combined with 34.5ENVE to synergistically kill ovarian cancer cells, and combination therapy results in prolonged survival and reduced tumor and ascites burden in the in vivo model of ovarian cancer. This may be a potential combination therapy for use in the clinic for patients with recurrent or refractive ovarian cancer.

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

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References


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