An oncolytic herpes simplex virus vector, G47Δ, synergizes with paclitaxel in the treatment of breast cancer

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Abstract. Paclitaxel-containing treatment regimens are standard chemotherapy schemes for breast cancer patients. The use of oncolytic herpes simplex virus (oHSV) vectors has been shown to be a safe and effective therapeutic approach for different types of cancer. We hypothesized that paclitaxel in combination with an oHSV vector would present an enhanced killing effect when used against breast cancer cells. In the present study, we demonstrated that the combined use of the oHSV vector G47Δ and paclitaxel produced a synergistic effect against breast cancer cells both in vitro and in vivo. In vitro studies demonstrated that paclitaxel and G47Δ both caused dose-dependent cytotoxicity against the human breast cancer cell lines MCF-7 and MDA-MB-468. G47Δ and paclitaxel also demonstrated synergistic cytotoxicity when applied together, with Chou-Talalay combination indices ranging from 0.44 to 0.77 for MCF-7 cells and 0.68 to 0.83 for MDA-MB-468 cells. Paclitaxel did not enhance viral replication or viral spread among tumor cells. However, G47Δ increased the antitumor ability of paclitaxel by inducing mitotic arrest and apoptosis. In vivo studies indicated that when combined with G47Δ, the dose of paclitaxel could be reduced at least 5-fold while maintaining levels of tumor reduction similar to those achieved with the administration of paclitaxel alone. Combination therapy resulted in no morbidity in vivo. Our data demonstrated that G47Δ and paclitaxel combination therapy had synergistic effects in the treatment of breast cancer. This combination therapy may be promising for breast cancer patients.

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related mortality in females (1). With the development of gene-expression profiling, researchers have found that breast cancer can be divided into different molecular subtypes with distinct clinical features and varying responses to therapeutic regimens (2-4). Patients with luminal A-type tumors may gain little benefit from chemotherapy. For those patients, hormone therapy may be sufficient for systemic treatment (5). However, chemotherapy remains the standard systemic treatment for other types of cancer, particularly human epidermal growth factor receptor 2 (HER-2)-positive tumors and basal-like tumors. Paclitaxel-containing drug regimens are the standard chemotherapy schemes for the treatment of breast cancer. Relative to anthracycline, paclitaxel significantly improves the disease-free survival and overall survival of patients; however, paclitaxel has a greater effect when used in conjunction with anthracycline (6-9).

The use of oncolytic viruses is a relatively new strategy in cancer therapy. Oncolytic herpes simplex virus (oHSV) vectors are inherently cytotoxic to and specific for tumor cells (10). The vector G47Δ was constructed as a third-generation replication-competent HSV-1 vector from HSV-1 laboratory strain F. The ICP47 gene and both copies of the γ34.5 neurovirulence gene are deleted in G47Δ, and the ribonucleotide reductase (RR) gene is inactivated by insertion of the E. coli LacZ gene (11). Due to these gene deletions/mutations, the replication ability of the virus is attenuated and, therefore, the safety and tumor selectivity are increased. The use of G47Δ has been shown to be a safe and effective therapeutic approach for various types of cancer (11-14).

Several preclinical studies have demonstrated that when oHSVs are used in combination with various cytotoxic agents, increased treatment efficacy for various malignancies is observed (15-18). GADD34 is a DNA repair enzyme that is homologous with the HSV-1 γ34.5 gene. Due to safety considerations, γ34.5 was deleted in the construction of G47Δ. This deletion attenuates the replication ability of HSV. Previous studies have demonstrated that chemotherapeutic agents that upregulate GADD34 in cancer cells in response to DNA damage enhance the antitumor effects of oHSV by facilitating viral proliferation (15-18). However, no published studies have investigated the mechanistic interactions between G47Δ and paclitaxel in the treatment of breast cancer.

Our previous preclinical studies showed that G47Δ can effectively treat primary breast tumors and brain and lung metastases (14,19,20). In the present study, we found that G47Δ...
and paclitaxel, when administered together, demonstrated a synergistic effect against the breast cancer cell lines MCF-7 and MDA-MB-468 in vitro. However, the use of paclitaxel did not enhance or impair viral proliferation. G47Δ replicated and spread among cancer cells, causing cancer cell lysis and enhancing paclitaxel activity. G47Δ facilitated the induction of mitotic arrest and apoptosis by paclitaxel. Finally, in vivo efficacy studies demonstrated that G47Δ and paclitaxel combination therapies are a safe and effective regimen for the treatment of breast cancer. When using paclitaxel in combination with G47Δ, the dose of paclitaxel could be reduced at least 5-fold while maintaining levels of tumor reduction similar to those achieved with the administration of paclitaxel alone. To the best of our knowledge, this is the first study to demonstrate that the combination of G47Δ and paclitaxel has synergistic effects in the treatment of breast cancer.

Materials and methods

Cells and viruses. MCF-7 and MDA-MB-468 cells (all obtained from Dr. Xiao-Ming Xie, Sun-sen University Cancer Center, China) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 4.5 g/l glucose. Vero cells (African green monkey kidney cells; American Type Culture Collection, Manassas, VA, USA) were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). Cells were cultured at 37°C and 5% CO₂. G47Δ was obtained from Samuel D. Rabkin (Molecular Neurosurgery Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA) and it was constructed as previously described (11).

Cell susceptibility assays and Chou-Talalay analysis. MCF-7 and MDA-MB-468 cells were seeded at 2,000 cells/well into 96-well plates. Following overnight incubation, paclitaxel and paclitaxel were added to the indicated concentration. To select the appropriate dose ranges, we used serial dilutions to test cell susceptibility. After 4 days of incubation, cytotoxicity assays were performed using a Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's instructions. Median effect doses (EDₜ₀) were calculated for the drug and G47Δ for each cell line.

For combination studies, G47Δ and paclitaxel were added to cells at a fixed dose ratio, and Cell Counting Kit-8 assays were performed after 4 days of incubation. To analyze the combination of G47Δ and paclitaxel, Chou-Talalay combination indices (CI) were calculated using CompuSyn software (Combio Syn, Inc., Paramus, NJ, USA). Fixed ratios of G47Δ and paclitaxel and mutually exclusive equations were used to determine the CIs. A CI between 0.9 and 1.1 is considered additive, whereas CI<0.9 and CI>1.1 indicate synergism and antagonism, respectively.

Viral proliferation. To determine whether paclitaxel enhances G47Δ proliferation, we tested the viral titers with or without the addition of paclitaxel. MCF-7 and MDA-MB-468 cells were seeded into 12-well plates at 2x10⁵ cells/well overnight and then treated with G47Δ at an MOI of 0.5 (MCF-7) or 1.5 (MDA-MB-468) and with paclitaxel at 1 nmol/l (MCF-7) or 3 nmol/l (MDA-MB-468). The cells and supernatants were collected for 4 days. After three freeze-thaw cycles, the titers of infectious virus were determined using a plaque assay with Vero cells. Next, we calculated the viral pfu per viable cancer cell. The cells were treated with G47Δ and a range of paclitaxel doses for 2 days. The viable cells were counted, and the viral titers were calculated.

Flow cytometric analysis of the cell cycle. MCF-7 and MDA-MB-468 cells were seeded into 10-cm dishes at 8x10⁵ cells/plate and treated with either the mock treatment, G47Δ, paclitaxel or the combination of G47Δ and paclitaxel. At the indicated time points, the adherent and detached cells were collected and washed in PBS. The cells were then washed twice in phosphate-buffered saline (PBS) containing bovine serum albumin (0.5%) and treated in 1 ml of PBS containing 0.1% Triton-X, RNase A (100 µg/ml; Sigma) and propidium iodide (PI) (50 µg/ml; Sigma) at room temperature for 30 min. The cells were then immediately analyzed by flow cytometry in a BD FACScanLib. The resulting data were analyzed with ModFit LT v3.2 (Verity Software House, Topsham, ME, USA).

Apoptosis assay. MCF-7 and MDA-MB-468 cells were seeded into 6-cm dishes at 4x10⁶ cells/plate and treated with either the mock treatment, G47Δ, paclitaxel or the combination of G47Δ and paclitaxel. Following 48 h of incubation, the adherent and detached cells were collected and washed twice with PBS. The cells were then counted, adjusted to a density of 1x10⁷/ml and double-stained with fluorescein isothiocyanate-conjugated Annexin-V and PI using an Annexin-V-FITC Apoptosis Detection Kit according to the manufacturer's instructions (Nanjing KeyGen Biotech., Co., Ltd., Nanjing, China). The stained cells were then immediately analyzed using flow cytometry with a BD FACScanLib. The resulting data were analyzed using FlowJo v8.5.3 software (Tree Star, Ashland, OR, USA).

In vivo treatment studies. First, 5x10⁶ MDA-MB-468 cells were implanted into the left flank of 6-week-old female BALB/c nude mice (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China). When the maximum diameters of the tumors reached ~5 mm, the mice were randomized into 5 groups (n=7 per group): a G47Δ treatment group treated with intratumor (i.t.) injections of G47Δ virus (2x10⁶ pfu) on Days 0 and 3, two paclitaxel treatment groups treated with intraperitoneal (i.p.) injections of paclitaxel (3 or 15 mg/kg) on Days 0 and 7 of a 7-day cycle for 2 weeks, a combination treatment group treated with G47Δ (2x10⁶ pfu) and paclitaxel (3 mg/kg), and a mock treatment group. The tumor volume was calculated using the formula width (mm) x length (mm) x 0.5. The body weight and motor activity of each animal were monitored as indicators of general health and toxicity.

Statistical analysis. The Student's t-test (two-tailed) was used to analyze the significance of differences between the treatment groups. The test was implemented using the SPSS version 13.0 software. A P-value of <0.05 was considered to indicate a statistically significant difference.
Results

G47Δ exhibits a synergistic effect in conjunction with paclitaxel in the killing of breast cancer cells in vitro. First, we performed cytotoxicity assays of paclitaxel and G47Δ with MCF-7 and MDA-MB-468 cells. Both the MCF-7 and MDA-MB-468 cells exhibited dose-dependent cytotoxicity following exposure to paclitaxel or G47Δ (Fig. 1). MDA-MB-468 cells were more sensitive than MCF-7 cells to paclitaxel at higher concentrations (4-32 nmol/l), although MDA-MB-468 cells were less sensitive at lower concentrations (0.5-2 nmol/l). MCF-7 cells were more sensitive than MDA-MB-468 cells to G47Δ cytotoxicity. The ED₅₀, i.e., the dose causing 50% cytotoxicity, was calculated for each agent. The ED₅₀ values of G47Δ were an MOI of 0.70 (MCF-7) and an MOI of 1.93 (MDA-MB-468). The ED₅₀ values of paclitaxel were 11.54 nm/l (MCF-7) and 5.90 nm/l (MDA-MB-468).

Next, we performed combination cytotoxicity assays at a fixed concentration ratio based on the above results. CIs were calculated to determine whether G47Δ and paclitaxel used in combination exhibit a synergistic breast cancer cell-killing effect in vitro. As defined, a CI between 0.9 and 1.1 indicates synergism and antagonism, respectively. The combination of G47Δ and paclitaxel produced a synergistic effect against both MCF-7 and MDA-MB-468 cells (Fig. 1). The Chou-Talalay CIs ranged from 0.44 to 0.77 for MCF-7 cells and from 0.68 to 0.83 for MDA-MB-468 cells. The most significant synergistic effect was achieved at moderate and high concentrations for MCF-7 cells and MDA-MB-468 cells, respectively. Overall, these results demonstrate that G47Δ and paclitaxel exhibit a synergistic anti-MCF-7 and anti-MDA-MB-468 effect in vitro when used in combination.

Paclitaxel does not affect the replication of G47Δ. To determine whether paclitaxel affects the replication of G47Δ in vitro, we calculated viral titers using plaque assays after infecting the cells with G47Δ and exposing the cells to paclitaxel or vehicle. The viral titers were tested daily. We found that paclitaxel did not significantly affect the replication of G47Δ (Fig. 2A and B). To extend these results, we next tested viral proliferation using different doses of paclitaxel and calculated the viral titer per viable cell. Similar to our previous results, the viral titers per viable cell did not significantly change across the different paclitaxel doses tested against MCF-7 or MDA-MB-468 cells (Fig. 2C and D). Finally, X-gal staining of MCF-7 cells infected with G47Δ and exposed to different doses of paclitaxel confirmed that paclitaxel did not significantly affect the spread of G47Δ (Fig. 2E).

G47Δ facilitates the induction of mitotic arrest by paclitaxel. Subsequently, we determined whether G47Δ affects the antitumoral activity of paclitaxel. Paclitaxel exerts its cytotoxic effect by interacting with β-tubulin, stabilizing the structure of microtubules and preventing the depolymerization of microtubules. This microtubule stabilization leads to cell cycle arrest in the G2/M phase and ultimately leads to cell death by apoptosis. We found that G47Δ alone did not significantly influence the cell cycle (Fig. 3). However, relative to treatment with paclitaxel alone, treatment with G47Δ plus paclitaxel significantly promoted cell cycle arrest in the G2/M phase after a 24-h exposure for both MCF-7 and MDA-MB-468 cells (Fig. 3).
For MCF-7 cells, the percentage of G2/M phase-arrested cells for the paclitaxel alone group and the combination group were 49.35±1.01% and 58.96±0.83% (P<0.001), respectively. For MDA-MB-468 cells, the percentages were 31.11±0.96% and 38.29±0.43% (P<0.001), respectively.

**G47Δ enhances the ability of paclitaxel to induce apoptosis in breast cancer cells.** We used two methods to assess the level of apoptosis. First, we calculated the percentage of sub-G0 cells after 72 h of exposure to the mock treatment, paclitaxel, G47Δ or a combination of paclitaxel and G47Δ. Relative to the mock treatment (1.17±0.12%), paclitaxel alone (15.70±0.95%), G47Δ alone (5.37±0.385%), and the combination of paclitaxel and G47Δ (24.29±0.60%) significantly increased the level of apoptosis in MCF-7 cells (P<0.05 for all comparisons) (Fig. 4A). Similar results were observed with MDA-MB-468 cells (Fig. 4B). Annexin-V and PI staining also confirmed that paclitaxel and G47Δ in combination produced a significant increase in apoptosis (Fig. 4C).

**G47Δ and paclitaxel exhibit synergistic effects in vivo.** Finally, we tested whether G47Δ and paclitaxel could exhibit a synergistic effect in vivo. We chose MDA-MB-468 cells to form tumors in BALB/C mice. After flank tumors developed, the mice were divided into 5 treatment groups. One group was treated with a mock treatment, another group received
i.t. G47Δ (2x10^5 pfu) injections on Days 0 and 3, two other groups received i.p. paclitaxel injections at different doses (3 or 15 mg/kg) twice a week for 2 weeks, and one group received a combination of G47Δ (2x10^5 pfu) + paclitaxel (3 mg/kg). We found that both G47Δ and paclitaxel could inhibit tumor growth in vivo. Compared with the mock treatment group, both the G47Δ and paclitaxel alone groups presented a significant reduction in the mean tumor volume by Day 35. In addition, we observed that the treatment of mice with a combination of G47Δ and paclitaxel resulted in a synergistic effect in vivo and a significant reduction in the mean tumor volume (68.71±22.65 mm^3) compared to treatment with G47Δ alone (277.57±75.35 mm^3), paclitaxel alone (501.86±74.79 mm^3), or the mock treatment (809.14±102.58 mm^3) by Day 35 (P<0.05 for all three comparisons) (Fig. 5A). Of note, when paclitaxel was combined with G47Δ, the dose of paclitaxel could be reduced at least 5-fold while maintaining levels of tumor reduction similar to those attained with the administration of paclitaxel alone at 15 mg/kg. We also found that the combination treatment produced a significant reduction in tumor weight compared with the other treatments (Fig. 5B). Furthermore, the combined treatment caused no additional signs of toxicity, and the body weight of the mice was similar among all groups (mean body weight, 22.5 g). In addition, the body weight did not change significantly over the course of the study.

**Discussion**

Breast carcinoma is a relatively low-malignancy type of tumor. Patients who suffer from early-stage breast cancer have positive clinical outcomes. However, approximately 30% of patients with early-stage disease eventually develop recurrent or metastatic lesions (21). At present, metastatic cancer remains an incurable disease. Therefore, new therapies are urgently required. Our group demonstrated previously that G47Δ effectively targets primary breast tumors and brain and lung metastases (14,19,20).

To increase safety and tumor selectivity, some genes were deleted from or mutated in the viral vector, including the RR gene and the γ34.5 neurovirulence gene. These changes attenuated the replication ability of HSV, resulting in oHSV being relatively selective for dividing tumor cells as there are cellular
homologs that can compensate for the missing genes, such as RR and GADD34 for γ34.5. Some preclinical studies have reported that oHSVs, when combined with chemotherapeutic agents that upregulate the expression or activities of RR and/or GADD34 in tumor cells, exhibit synergistic effects against various tumors (15-18). Petrowsky et al (15) reported that fluoro- deoxyuridine, which causes nucleotide pool imbalances and DNA damage-induced upregulation of RR and GADD34 in colorectal cancer cell lines, promotes enhanced viral replication and tumor cell death.

In the present study, we demonstrated, using Chou-Talalay assays, that G47Δ and paclitaxel in combination exhibited a synergistic effect against MCF-7 and MDA-MB-468 breast cancer cells. We initially hypothesized that paclitaxel might enhance viral replication. However, both viral titer experiments and X-gal staining indicated that paclitaxel did not significantly influence viral replication or spread. These results indicated that the mechanism mediating the synergistic cytotoxicity between G47Δ and paclitaxel does not involve the enhancement of viral replication by paclitaxel.

To further investigate the interaction between G47Δ and paclitaxel, we determined whether G47Δ enhanced the anti-tumor activity of paclitaxel. Paclitaxel stabilizes the structure of microtubules, leading to cell cycle arrest in the G2/M phase and ultimately inducing cell death by apoptosis. First, we found that although G47Δ alone did not significantly impact the cell cycle of either MCF-7 or MDA-MB-468 cells, G47Δ and paclitaxel, when administered in combination, resulted in significantly greater numbers of tumor cells arrested in the G2/M phase, compared to treatment with either paclitaxel or G47Δ alone. Then, we found that both G47Δ and paclitaxel could cause tumor cell apoptosis, but G47Δ alone only produced a small percentage of apoptotic cells. However, G47Δ significantly enhanced the ability of paclitaxel to induce apoptosis, relative to paclitaxel alone. Elliott and O’Hare (22) found that the HSV-1 tegument protein VP22 can reorganize microtubules into thick bundles that become highly resistant to microtubule-depolymerizing agents, suggesting that VP22 might have the capacity to stabilize the microtubule network. Our data indicate that G47Δ administered by itself at a low dose did not significantly influence the cell cycle of MCF-7 or MDA-MD-468 cells, but VP22 might enhance the ability of paclitaxel to stabilize the structure of microtubules, eventually leading to G2/M phase arrest and cell apoptosis.

Although paclitaxel is used widely to treat a variety of tumors, the toxic effect of the drug is a key factor in restricting its broader clinical use. Hematopoietic toxicity and cumulative peripheral neuropathy limit the long-term use of paclitaxel, which always leads to dose reduction and the delay of paclitaxel chemotherapy, particularly for patients with recurrent or metastatic disease, most of whom have been heavily treated for the primary disease. Kim et al (23) found that when breast cancer patients received adriamycin, cyclophosphamide and paclitaxel as adjuvant chemotherapy for their primary tumors, the drug dose was reduced for 17.1% of patients, and 14.3% patients delayed treatment due to the toxicity of the chemotherapy agents. Loibl et al (24) observed that patients receiving a lower relative total dose had a shorter overall survival. In the present study, we demonstrated that G47Δ and paclitaxel combination therapy can reduce the required paclitaxel dose by at least 5-fold while maintaining levels of tumor reduction similar to those achieved with the administration of paclitaxel alone. In addition, the combination therapy caused no additional signs of toxicity. These data may have important clinical implications. For some patients, especially patients who have ever been heavily treated for recurrent or metastatic disease, G47Δ and paclitaxel combined therapy may be an effective and safe therapeutic regimen.

In summary, we report that a third-generation replication- competent HSV-1 vector G47Δ and paclitaxel, when given in combination, had a synergistic anti-breast cancer effect both in vitro and in vivo. Paclitaxel did not significantly influence the replication and spread of G47Δ, but G47Δ may enhance the antitumor activity of paclitaxel through mitosis arrest and apoptosis. G47Δ and paclitaxel combined therapy appears to be an effective and safe therapeutic regimen for the treatment of breast cancer, and, thus, the presented data may have critical clinical implications for the treatment of breast cancer. To the best of our knowledge, this is the first report demonstrating a synergetic effect of a combination of G47Δ and paclitaxel against breast cancer cells.
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References