



DOI: 10.19187/abc.20185290-95

## Targeting Breast Cancer With Bio-inspired Virus Nanoparticles

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### ARTICLE INFO

**Received:**  
16 April 2017  
**Revised:**  
4 June 2017  
**Accepted:**  
5 June 2017

**key words:**

Breast cancer cell lines,  
cytotoxicity,  
Herceptin,  
plant viral nano  
particles,  
*Potatovirus X* (PVX)

### ABSTRACT

**Background:** Viral nanoparticles are biodegradable, biocompatible, self-assembling, and highly symmetric, and can be produced in large quantities. Several plant viral nanoparticles (VNPs) have been exploited in different areas of nanobiotechnology, especially drug delivery in cancer therapy. In this study, a flexuous plant virus called potato virus X (PVX) is presented with a unique nanoarchitecture which can increase tumor homing and penetration. Thus, this study aimed to investigate the potential of PVX for delivering Herceptin (HER) in different breast cancer cells and normal cells.

**Methods:** PVX was conjugated to HER by EDC/Sulfo-NHS in two steps. After confirming the conjugation, PVX-HER efficacy and drug activity were investigated in HER2-positive (SKBR3 and SKOV3), HER2-negative (MCF-7 and MDA-MBA-21), and non-tumorigenic epithelia breast cancer (MCF-12A) cell lines after treatment with 10 and 20 µg of the drug. Then, PVX-HER was imaged in SKBR3 cells in to study the nuclear accumulation of the drug at different concentrations.

**Results:** An increased cytotoxic efficiency was observed for PVX-HER vs free-HER in SKBR3 and SKOV3 cell lines. However, the efficacy of PVX-HER failed to increase in MCF-7, MDA-MB-231, and MCF-12A cell lines compared with free-HER after 24 hours. In addition, compared with free-HER, Herceptin nuclear accumulation was increased in SKBR3 cells treated with PVX-HER. Further, the PVX-HER treatment resulted in reduced tumor growth in the HER2-positive cells lines. Finally, a direct relationship was observed between the imaging results and MTT assay in SKBR3.

**Conclusion:** PVX-HER displays a significantly greater cytotoxic activity compared with free-HER in HER2-positive cells.

### Introduction

Nanotechnology is one of the emerging sciences which has attracted a lot of attention in medicine.<sup>1</sup> Generally, nanoparticles are defined as molecules measuring 1 to 100 nanometers. Nanoparticles represent a remarkable structural diversity including dots, nanotubes, wires, and capsuls.<sup>2</sup> In recent years,

the use of nanoparticles in medicine has increased, contributing greatly to reductions in effective drug dose, drug side effects, cost, and toxicity.<sup>3</sup>

Nowadays, different nanoparticles have been identified, and most of them are chemical. There are currently various groups of nanoparticles used in medicine, including synthetic and biological nanomaterials. Each of these particles has advantages and disadvantages<sup>4</sup> Viral nanoparticles (VNPs) are naturally occurring bionanomaterials based on pathogenic or nonpathogenic bacterial, plant, or animal viruses. VNPs have self-assembling systems that enable them to proliferate in large quantities within a short time. In addition, they are highly symmetrical, biocompatible, and biodegradable.

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VNPs from plants are preferable to those from animals due to their safety.<sup>5</sup> One of the most popular rod-shaped VNPs is potato virus X (PVX), which is considered an important economic filamentous virus of  $515 \times 12$  nm in size belonging to the genus *Potexvirus* and the family *Alphaflexiviridae*. The virus encompasses economically important pathogens worldwide, with variable symptoms. PVX has a single-stranded positive-sense RNA genome which is approximately 6400 nucleotides long with a cap and poly(A) tail. The genome contains five open reading frames (ORFs) which encode proteins of 166, 25, 12, 8, and around 26 kDa, respectively. Esfandiari *et al.* reported a new isolate of PVX (PVX-Iran, accession no. FJ461343) (figure 1).<sup>6</sup> Over the last decade, virus research has been led toward the beneficial use of viruses independent of their potential pathogenesis.<sup>7</sup>

PVX nanoparticles are used for imaging, drug delivery, and diagnosis.<sup>8</sup> Further, PVX could increase tumor homing and penetration, compared to spherical cowpea mosaic virus (CPMV).<sup>9</sup> Conjugation of PVX to doxorubicin (PVX-DOX) caused a reduction in tumor growth.<sup>10</sup>

The present study aimed to evaluate the PVX for drug delivery in targeted therapy. In addition, based on the results, the PVX-Iran conjugated to Herceptin (PVX-HER) increased the death of two breast cancer cell lines (SKBR3 and SKOV3).<sup>11</sup>

Herceptin (trastuzumab) is an FDA-approved antitumor for breast cancer. It is a humanized monoclonal antibody which can be regarded an effective treatment for HER2-positive breast cancer.<sup>12</sup> Breast cancer is the most common type of cancer among women.<sup>13,14</sup> The present study focused on the use of PVX for delivering Herceptin in HER2-positive, HER2-negative, and normal cells. Herceptin was loaded onto the surface of an isolated PVX6 particles (PVX-HER) by EDC/Sulfo-NHS and accordingly the efficacy of the approach had been examined in SKBR3 and SKOV3 after characterization.<sup>11</sup> In the present study, two triple-negative breast cancer cell lines (MDA-MB-231 and MCF-7), along with MCF-12A non-tumorigenic mammary epithelial cell line, as control normal cells, were examined. Finally, tracking the free-HER and PVX-HER were evaluated in the cells using fluorescent microscopy.



**Figure 1.** Genome organization of PVX-Iran and the proteins encoded by the genome

## Methods

*Nicotiana benthamiana* is a common experimental host in propagating PVX in large amounts. *N. benthamiana* leaves were inoculated with phosphate buffer (pH 7.2) for the purpose of purification, which caused an increase in virus accumulation after 2 to 3 weeks, and the leaves were harvested and purified as described previously.<sup>15</sup>

To prepare PVX-HER nanoparticles, we incubated PVX ( $5 \mu\text{g } \mu\text{l}^{-1}$ ) with an EDC/Sulfo-NHS (N-hydroxysulfosuccinimide) cross-linker for 4 hours in reaction buffer (0.5 M NaCl, 0.1 M MES, pH 6). In order to remove the unconjugated free linker, the reaction was quenched by 2-mercaptoethanol and Biogel P-10 column (Bio-Rad). Accordingly, HER ( $50 \mu\text{g } \mu\text{l}^{-1}$ ) was conjugated to PVX-EDC/Sulfo-NHS for 2 h in 0.1 M NaCl buffer (pH 7.5). Then, PVX-HER nanoparticles were purified by a Biogel P-10 column (Biorad).<sup>11</sup> In the present study, the characterization was conducted using Zetasizer (ZEN 3600, Malvern) and also with Western blotting using a PVX-specific primary antibody (DSMZ, PV-0027)

and a secondary goat polyclonal antibody against rabbit IgG-HRP (Abcam, ab6721).

## Cell Culture

SKBR3 and SKOV3, as HER2+ cells lines, and MDA-MB-231 and MCF-7 (as HER2- cells lines) were cultured in RPMI-1640 medium (RPMI, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin-streptomycin (Pen-Strep, Gibco). Then, MCF-12A cells as a normal breast cancer was grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) FBS, 1% (v/v) pen-strep. Finally, all cells were seeded in 96-well plate (SPL, Life Sciences, Gyeonggi, Korea) and incubated at 37°C and 5% CO<sub>2</sub> in a humidified environment.

## Cytotoxicity Evaluation

Cells were washed with PBS after reaching a minimum 75% confluence. To this end, they were trypsinized with 0.25% (w/v) trypsin-EDTA (Gibco,



Germany) and seeded at  $5 \times 10^3$  cells per well in a 96-well flat-bottomed plate. Following 24 h incubation at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ , the cells were washed with PBS and treated with free-PVX and PVX-HER at HER concentrations of 10 and 20  $\mu\text{g}$ . After incubation for 24 h, an MTT assay was done according to the manufacturer's protocol. Briefly, 50 mM of MTT solution was added to each well, and the plate was incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  for 4 h. Then, the MTT dye was reduced at the viable cells, developing purple crystals. The MTT solution was then removed and the cells were suspended in 200  $\mu\text{l}$  DMSO in order to dissolve formazan crystals, and the plate was read at 570 nm. Finally, the data were normalized to the untreated control wells for each experiment. The assays were performed in triplicate and repeated in three independent experiments.

#### Tracking of PVX-HER in Cells

First, HER2/neu-positive breast cancer cells (SKBR3) were cultured. After reaching the cells confluency, they were seeded onto coverslips (SPL, 20012) in 24-well plates ( $5 \times 10^4$  cell/well) and incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . In the next step, cells were washed and treated with free-HER (10  $\mu\text{g}$ ) and PVX-HER (10 or 20  $\mu\text{g}$ ). After 4 hours, the cells were washed, fixed in cold solution containing methanol/ethanol (1:1), and washed with PBS. Additionally, 4% BSA in 1X PBS was used for 30 min at room temperature. PVX antibody (DSMZ,

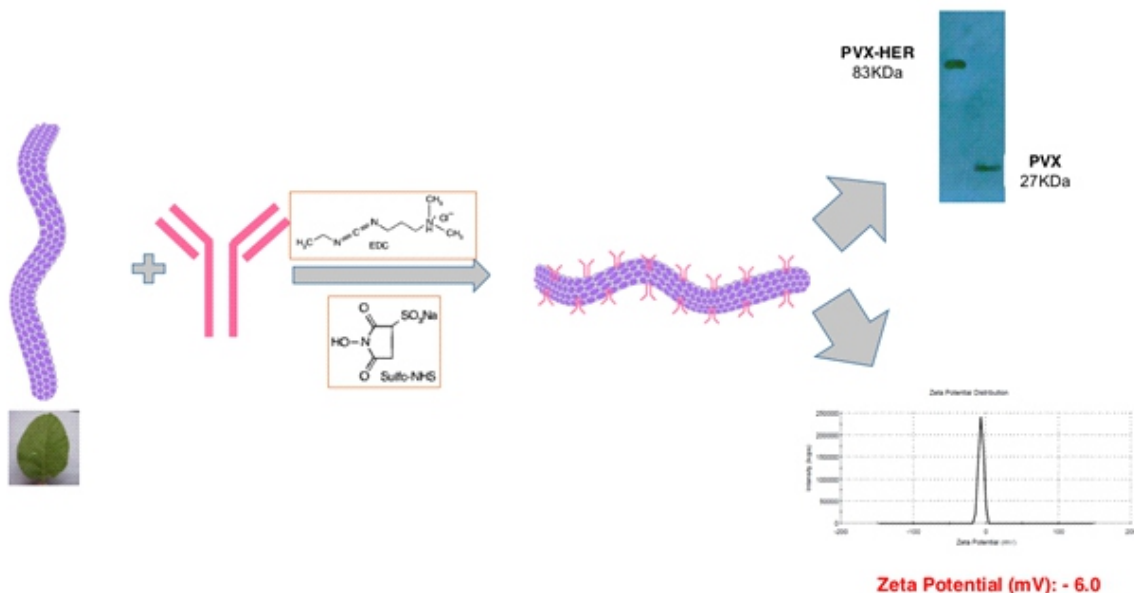
PV-0027) was added and shaken for 90 minutes. Finally, the coverslips were washed with PBST (phosphate-buffered saline with Tween-20) and mounted by donkey anti-rabbit IgG-PE (Santa Cruz, sc-3745). The slides were imaged with a LABEX fluorescent inverted microscope.

#### Statistical Analysis

Data were analyzed using analysis of variance (ANOVA), along with LSD post hoc test, on SPSS 16.0. Results are reported as mean  $\pm$  standard error of mean (SEM). Before determining the level of significance for the tests, the normality of data distribution was tested using Kolmogorov-Smirnov and Shapiro-Wilk tests. The difference in values was significant if the P value was less than 0.001. All experiments were performed in triplicate and repeated at least 3 times.

#### Results

After conducting the mechanical inoculation of *N. benthamiana*, mosaic symptoms were observed within 3 weeks. About 1 mg of pure PVX was extracted from each gram of infected plant. Western blotting with human antibody demonstrated a protein band of HER with a molecular weight of 55 kDa and PVX-HER conjugate with a molecular weight of 83 kDa. Also, the value obtained by Zetasizer for HER-PVX was -6.0 that was between PVX (-21.4) and HER2 (-1.48) charges (figure 2).



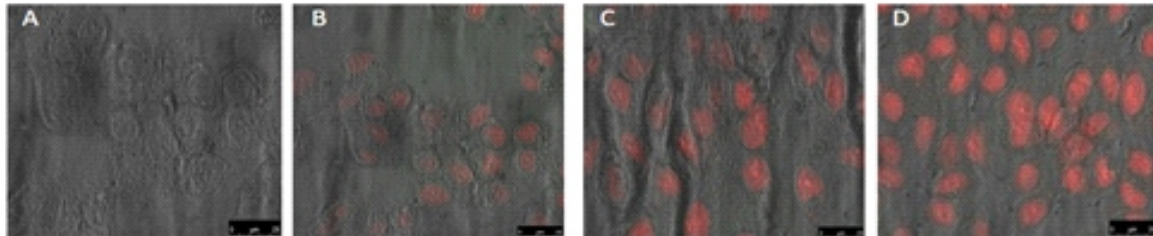
**Figure 2.** Scheme of potato virus X (PVX) conjugated to Herceptin (HER) with EDC/Sulfo-NHS in a two-step protocol western blot with PVX-specific antibody (DSMZ, PV-0027) and zeta potential was used to confirm conjugation.

In the present study, cytotoxic activity of HER-PVX against HER2-positive, HER2-negative, and normal cell lines was examined. SKBR3, SKOV3, MDA-MB-231, MCF-7, and MCF-12A cells were

treated with 10  $\mu\text{g}$  and 20  $\mu\text{g}$  of PVX-HER and free-HER for 24 hours. The results of cell viability assay are presented in Table 1.

**Table 1.** The mean percentage  $\pm$  SEM of cell viability for breast cancer cell lines by MTT

	PVX-HER (10 $\mu$ g)	Free-HER (10 $\mu$ g)	PVX-HER (20 $\mu$ g)	Free-HER (20 $\mu$ g)
SKBR3	17.973 $\pm$ 0.005	47.697 $\pm$ 0.004	16.067 $\pm$ 0.004	42.510 $\pm$ 0.003
SKOV3	28.003 $\pm$ 0.005	37.641 $\pm$ 0.004	22.916 $\pm$ 0.006	33.518 $\pm$ 0.002
MCF-7	96.322 $\pm$ 0.010	98.478 $\pm$ 0.009	96.569 $\pm$ 0.0108	95.859 $\pm$ 0.0135
MDA-MB-231	97.420 $\pm$ 0.009	97.856 $\pm$ 0.011	96.889 $\pm$ 0.005	96.661 $\pm$ 0.0092
MCF-12A	98.590 $\pm$ 0.010	98.561 $\pm$ 0.011	99.059 $\pm$ 0.006	98.559 $\pm$ 0.009

**Figure 3.** Nuclear accumulation of Herceptin in SKBR3 after 3 hours of incubation (A) control, (B) free-HER 10  $\mu$ g, (C) PVX-HER 10  $\mu$ g, and (D) PVX-HER 20  $\mu$ g.

In addition, fluorescence microscopy was performed to monitor Herceptin accumulation in the cells in order to investigate the efficacy of PVX-HER compared with free-HER. SKBR3 cells were treated with free-HER (10  $\mu$ g) and PVX-HER (10  $\mu$ g and 20  $\mu$ g) for 4 hours. Herceptin accumulated inside the nuclei of SKBR3 cells after 3 hours (figure 3). Localized delivery of Herceptin in the nucleus is more than that of cytoplasm.<sup>16</sup> Fluorescence intensity is positively correlated with nuclear Herceptin accumulation. Thus, the greater fluorescence intensity in the cells treated with PVX-HER compared with free-HER indicates the greater efficacy of the PVX delivery system.

### Discussion

The use of VNPs in medicine has expanded rapidly over the past 20 years to span a wide range of applications from imaging to targeted drug delivery. VNPs are naturally occurring bionanomaterials with unique symmetrical outer surface.<sup>17</sup> They are biodegradable, nontoxic for mammals,<sup>18</sup> and are produced at lower costs.<sup>19</sup> Some studies have emphasized their use as contrast agents in MRI imaging and as facilitators in drug delivery.<sup>5,20</sup>

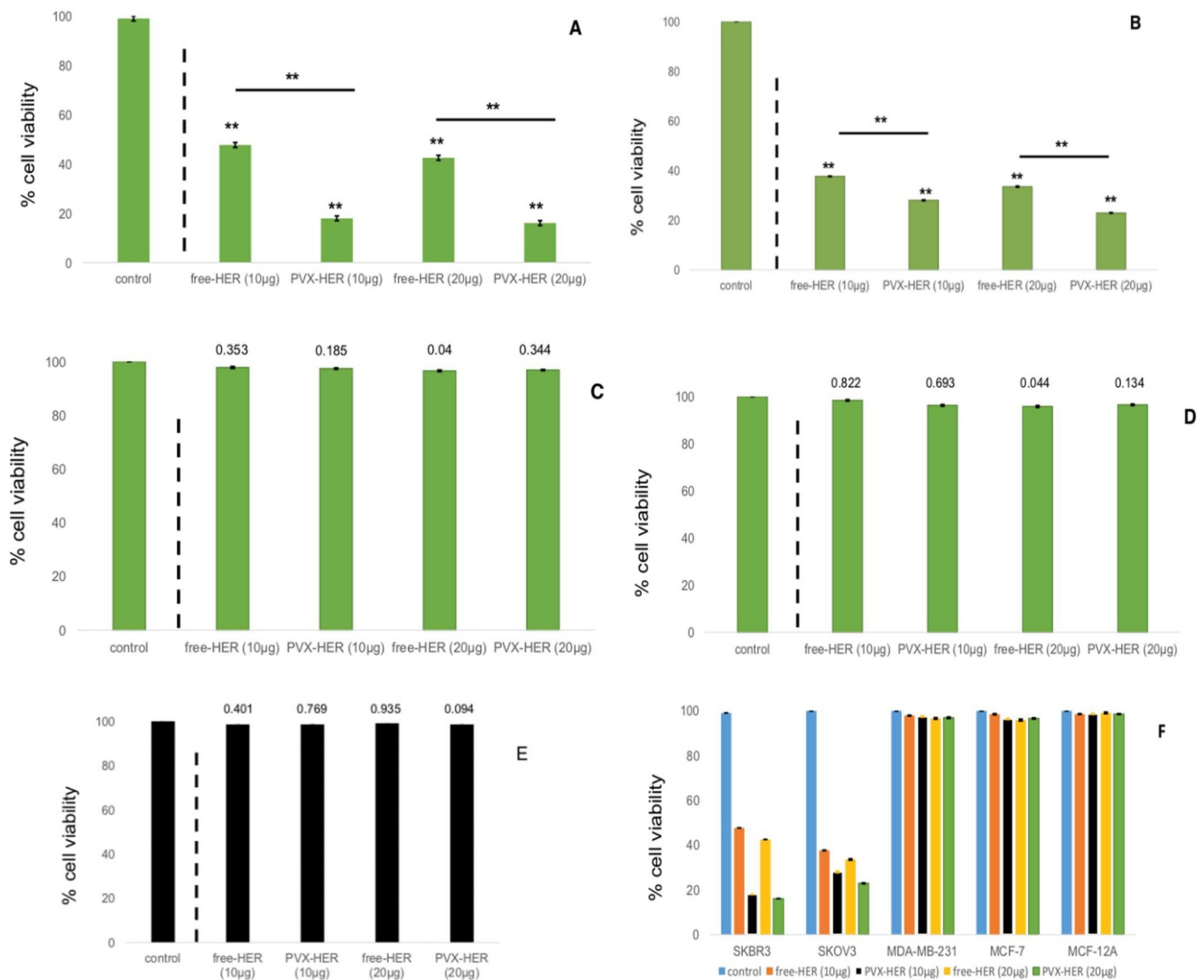
PVX is the type member of the genus *Potexvirus* with flexible rod particles measuring 515  $\times$  13 nm.<sup>21</sup> PVX has proved more effective in tumor homing and penetration compared with spherical or isometric virus.<sup>20</sup>

During recent years, the benefits of PVX have increased the use of this virus as a VNP, which have been emphasized in different cases such as the use of PVX for drug delivery system for Doxorubicin (DOX)<sup>10</sup> and Herceptin for cancer therapy.<sup>11</sup> The development of coadministered PVX-DOX in situ vaccination for treatment is a possibility.<sup>22</sup>

Herceptin is the trade name for trastuzumab, approved by the FDA in 1998 in order to treat human

epidermal growth factor receptor (HER) 2-positive breast cancer. Herceptin is an expensive monoclonal antibody which can cause serious side effects.<sup>23</sup> The conjugation of drugs to nanoparticles helps to enhance therapeutic efficacy and reduce side effects.<sup>24</sup>

In the previous study, PVX-HER nanoparticles significantly increased the death rate of HER2-positive cancer cells (SKBR3 and SKOV3). In the present study, the effect of this nanoparticle on other cell lines was investigated to complete the previous information. Thus, the effect of PVX-HER on cell viability was tested in SKBR3 (breast cancer) and SKOV3 (ovarian cancer) as HER2-positive cells, MDA-MB-231 (breast cancer) and MCF-7 (breast cancer) as HER2-negative cell lines, and MCF-12A as a nontumorigenic epithelial breast cell line. The results of ANOVA indicated a significantly increased cell death in SKBR3 (figure 4-A) and SKOV3 (figure 4-B) cell lines when treated with PVX-HER compared with free-HER. However, in MDA-MB-231 (figure 4-C), MCF-7 (figure 4-D), and MCF-12A (figure 4-E) cell lines, there was no significant difference in the mean absorption values between PVX-HER and free-HER. Therefore, the results provide a clear answer to how PVX-HER works on breast cancer cells. Further, SKBR3 cells were expected to address the question about the uptake of PVX-HER in the cells based on the results obtained by MTT assay. Fluorescence microscopy was used to monitor nuclear accumulation of Herceptin. As reported in previous studies, Herceptin accumulated within the nuclei of the SKBR3 cells after 3h.<sup>16</sup> Then, a greater nuclear accumulation of Herceptin was observed in SKBR3 cells treated with 10 and 20  $\mu$ g of PVX-HER compared with 10  $\mu$ g of free-HER, indicating the greater efficacy of the PVX-based drug delivery systems.



**Figure 4.** In vitro cell viability were determined using MTT assay after 24 h treatment with free-HER (10 µg), PVX-HER (10 µg), free-HER (20 µg), PVX-HER (20 µg), and control. Data represent normalized mean values in SKBR3 (A), SKOV3 (B), MDA-MB-231 (C), MCF-7 (D), and MCF-12A (E) cell lines. Asterisks indicate significant difference between free-HER and PVX-HER in SKBR3 (A) and SKOV3 (B) cell lines  $**P \leq 0.001$ . In the case of insignificant differences, the P values are mentioned on the columns in the MDA-MB-231 (C), MCF-7 (D), and MCF-12A (E). Cell viability in control (blue), free-HER 10 µg (orange), PVX-HER 10 µg (black), free-HER 20 µg (yellow), PVX-HER 20 µg (green) on SKBR3, SKOV3, MDA-MB-231, MCF-7, MCF-12A cell lines (F).

PVX-HER displayed a significantly greater cytotoxic activity compared with free-HER in HER2-positive cells. Therefore, the relationship between the imaging results and the MTT assay following 24 hours of incubations with 10 and 20 µg of PVX-HER may be explained. However, PVX-HER failed to increase the cytotoxic efficacy in HER2-negative and normal epithelial breast cell lines compared with free-HER (figure 4-F).

In general, based on the drug delivery features of PVX, the results of the present study were consistent with the findings of the previous studies on VNPs. Doxorubicin was covalently conjugated to CMV,<sup>25</sup> CPMV,<sup>26, 27</sup> PVX,<sup>10</sup> and RCNMV<sup>28</sup> in order to enhance the drug delivery.

The results of this study, as the first research on

PVX-HER nanoparticles, together with that of the previous study, confirm the effect of PVX-HER nanoparticles on breast cancer cells.

As conclusion, the present study recommends the use of the new isolated PVX, as a plant viral nanoparticle, for delivering Herceptin. Based on the results, Herceptin loaded onto the surface of PVX increases the drug efficacy in HER2-positive cancer cells. The results can contribute to development of filamentous plant virus-based drug delivery systems in cancer treatment.

#### Acknowledgement

The research was in part supported by Shahid Beheshti University. The author gratefully acknowledges the helpful suggestions of the anonymous referees.



### Conflict of Interest

The author declares no conflict of interests.

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