Precise Phage-navigating Breast Cancer-targeted Paclitaxel-Micell Nanomedicines

Valery A. Petrenko* & Vladimir P. Torchilin**

*Department of Pathobiology, College of Veterinary Medicine, Auburn University, Alabama, USA, petreva@auburn.edu

**Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, Boston, MA, USA, v.torchilin@neu.edu

ABSTRACT

Breast cancer is one of the most common cancers in the USA. A common cause of breast cancer mortality is *metastasis* to lymph nodes, lung, liver, bone and brain. The widely used drug for the treatment of metastatic breast cancer (MBC) is paclitaxel (PTX). Beside its high anticancer efficacy, clinical applications of PTX are limited because of its insolubility in water and complications in patients,. To enhance therapeutic efficacy and decrease side effects of PTX, we encapsulated the drug in micelles and targeted them towards the tumor cells. Using our proprietary "Landscape Phage Technology", we developed "Paclitaxel-PEG-PE Micells, Targeted to Breast Cancer cells by Tumor-Specific Landscape Phage Fusion Proteins" shortly—"*P-micelles*". Due to improved tumor delivery of paclitaxel, *P-micelles* showed selective toxicity to cancer cells *in vitro* and triggered a dramatic tumor reduction and extensive necrosis *in vivo*. The increased anticancer effect *P-micelles* was verified by enhanced apoptosis and reduced tumor cell proliferation. The absence of hepatotoxicity and pathologic changes in tissue sections of vital organs, together with maintenance of overall health of mice following the treatment, further supported its translational potential as an effective and safe chemotherapy for improved breast cancer treatment

Keywords: targeted nanomedicines, micelles, paclitaxel, breast cancer, landscape phage, major coat protein

1 INTRODUCTION

Despite the increase of 5-year relative survival rate of women with breast cancer for the last decades, it is still low (~26%) in patients diagnosed at late stages of disease [4]. A common cause of breast cancer mortality is metastasis to lymph nodes and organs. One of the most widely used drug for the treatment of MBC is paclitaxel (PTX). Beside its anticancer efficacy, clinical applications of PTX are limited due to its negative side effects in breast cancer patients. Furthermore, the serious complications, such as resistance to chemotherapy and subsequent progression of disease are observed in metastatic patients. To enhance therapeutic efficacy and decrease side effects of cancer therapeutics in patients, their encapsulation and targeting of the resulting nanomedicines towards the vasculature and tumor cells has been proposed. However, despite the initial promise, targeting of PTX preparations, as other nanomedicines, still has not demonstrated significant benefit at the preclinical or clinical level. To respond to the evolving concern regarding the use of targeted nanomedicines in human patients [6, 7], we proposed a new class of '*phage-programmed drug-delivery vehicles'* with tissue-travelling capacities, able to overwhelm and go through biological barriers surrounding tumor. In this *multi-targeting strategy, promiscuous fusion phage proteins* serve as *navigating ligands*, which interact with a repertoire of vasculature, tumor and tumor-surrounding cells, and bring their cargo, such as PTX, to a certain internal compartment of the cancer cells. Here, we illustrate the performance of this approach for development of efficient breast cancer nanomedicines.

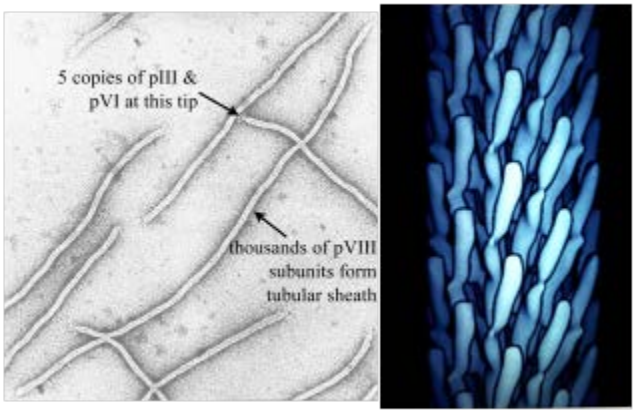

Fig. 1. Electron micrograph (left) and electron density model (right) of filamentous phage. The aminoterminus of pIII proteins are visible and pointed by arrow (adapted from [1]).

2. PACLITAXEL-LOADED PEG-PE-MICELLAR NANOMEDICINES TARGETED WITH TUMOR SPECIFIC LANDSCAPE PHAGE FUSION PROTEIN

The utility of polymeric micelles offers an efficient solution for the solubilization and tumor-targeted delivery of a variety of sparingly soluble therapeutic agents [8, 9]. Polymeric micelle formulations currently under clinical investigation include PEG-PLA micelles loaded with paclitaxel (Genexol-PM) [10]. Clinical data have shown that these micellar formulations have improved half-life, increased bioavailability and reduced toxicity. Additional

improvement of the tumor-targeted efficiency of micellar drugs can be achieved by the surface modification of a micellar formulation with tumor-specific ligands, which selectively recognize tumor cell-associated antigens or receptors [11].

2.1 landscape phage libraries—a reservoir for construction of tumor-targeted nano-vehicles

Most targeted drug delivery projects use phage-display systems that explore filamentous phages—long thin viruses, consisting of a single-stranded circular DNA packed in a cylindrical shell composed of the major coat protein pVIII (MCP) (~90% of the virion mass), and a few copies of minor coat proteins capping the ends of the phage particle (**Fig.1**). In phage display constructs, a foreign coding sequence is spliced in-frame into one of the phage coat protein genes, so that the "guest" peptide encoded by that sequence is fused to the phage coat protein and thereby displayed on the exposed surface of the virion [12]. A phage display library is an ensemble of up to about 10 billion such phage clones, each displaying a different guest peptide on the virion surface. The minor coat protein pIII and the major coat protein pVIII, commonly used for phage display, are presented by 5 copies at the phage distal end and up to 2700 copies all over the virus surface (~4,000 copies in fd-tet type vectors used in this project) respectively (**Fig.3A,B**). PIII-displayed libraries are used to select high affinity peptides and antibodies in affinity selection procedure called biopanning. In contrast, the pVIII-expressing phages, such as landscape phage libraries, used in this project, allow selecting peptides with lower affinities, as their dense arrangement on the virion's surface results in a stronger binding due to avidity, masking low individual peptide affinity.

2.2 Molecular toolkit for design of P-micelles

The major construction material that we used in the preparation of **P-micelles** was the **Major Coat Protein pVIII (MCP)** of landscape phages [1]. Proficiency of the MCP to associate with micelles and liposomes [13, 14]

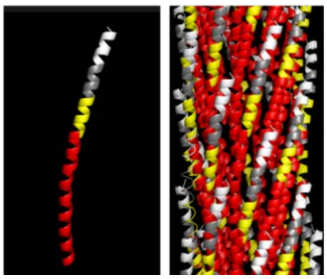

Fig.2A.Three-dimensional helical structure of pVIII coat protein (**left**) the landscape phage (~1% fragment, **right**). **White** segment depicts guest peptides. **Yellow** area shows segment that can be mutated.

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fd      AEGDDPAKAAFDSLQASATEYIGYAWAMVVVIVGATIGIKLFKKFTSKAS-50
f8/8:   AXXXXXXXXDPAKAAFDSLQASATEYIGYAWAMVVVIVGATIGIKLFKKFTSKAS-55
f8/9:    AXXXXXXXXXPAKAAFDSLQASATEYIGYAWAMVVVIVGATIGIKLFKKFTSKAS-55
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Fig.2B. MCP pVIII in phage fd and polyvalent f8-type fusion-peptide phage-displayed libraries (Landscape Libraries). **Blue X** – Random amino acid. **Green** –sites that have been mutagenised during affinity maturation of phage.

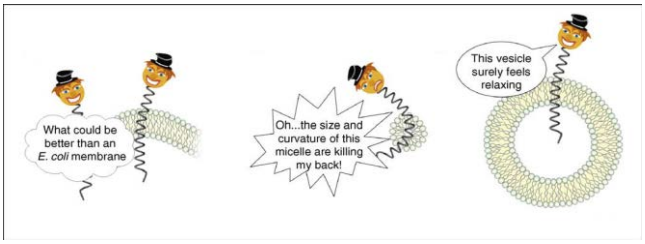

Fig. 3. Cartoons illustrating accommodation of the transmembrane MCP pVIII protein: (left) natural in vivo environment; (center) Detergent micelles; (right) Diluted vesicular membrane systems. Adapted from [3].

emerges from its intrinsic function as a membrane protein during infection of the host *E. coli* and later - during the phage assembly [3, 15] (**Fig.3,4**). Spontaneous insertion of the major coat protein into lipid membranes is mediated by electrostatic, electrophoretic, and hydrophobic interactions, as discussed in [1]. Along with the use of the major coat protein for targeting of liposomes and micelles, fusion phage proteins have been used for encapsulation of DNA and RNA [1, 16]. This approach mimics the mechanism of encapsulation of the phage DNA during phage proliferation [17]. In our experiments, landscape phage libraries f8/8 and f8/9 have been used to discover phages specifically interacting with various type cancer cells [1, 18-20]. It was demonstrated that phage fusion proteins selectivity interacting with various cellular phenotypes are ideal construction material for preparation of targeted nanomedicine platforms with increased anti-tumor potential towards human prostate, breast, lung and pancreatic cancer cells [21-23]. It was shown that the cell-binding specificity of selected phages and their proteins translates to the protein-decorated nanomedicines, enhancing their binding and cytotoxic potencial towards the cancer cells. The major principle of the landscape phage-based approach is that targeted nanomedicines recognize the same receptors on the

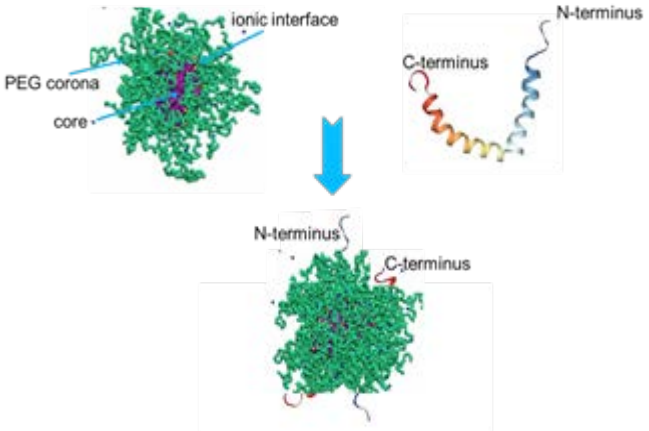

Fig.4. Insertion of the fusion MCP into 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N [Methoxy (polyethylene glycol) 2000] (**DSPE-PEG2000**). Elements of the scheme adapted from [2]). In the equilibrated micelles, we can recognize three unique regions: core, ionic interface, and PEG corona.

target cells, which were displayed in the phage selection procedure.

We screened such a landscape phage fusion protein pVIII specific for breast cancer MCF-7 cells [24-27]. Its self-assembly with 1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine-N-[methoxy(polyethyleneglycol)-2000 (**PEG_{2000}-PE**) conjugates produced micellar nanoparticles capable for the delivery of hydrophobic PCT to specific tumor cells [28]. Our *in vitro* results supported the concept that MCF-7-targeted PCT P-micelles demonstrated enhanced binding and cytotoxicity against MCF-7 breast cancer cells. We assessed the effect of the targeted therapy on cellular apoptosis, necrosis and proliferation as well as its *in vivo* antitumor efficacy and potential side-effects.

2.3 Construction and preclinical study of phage-navigating breast cancer paclitaxel-micelles

The landscape phage-based technology opened the way for using easy-to-prepare fused phage proteins as "substitute antibodies" for site-specific targeting of drugs and drug-loaded pharmaceutical nanocarriers. It allows converting the selected phages into targeted cytotoxic drugs in three steps: **a)** stripping of selected phages; **b**) isolation of their major

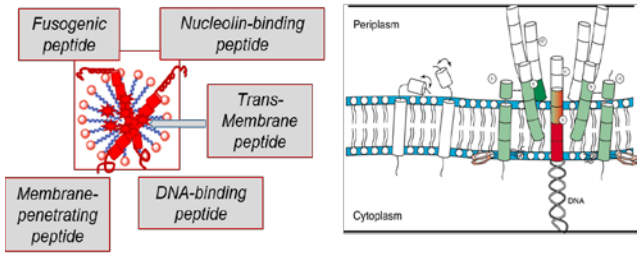

Fig.5. Association of the major coat protein pVIII with micelles emerging from its intrinsic function as a membrane protein during phage assembly: A: Multifunctional nucleolin-targeted micellar paclitaxel. B: Incorporation of major coat proteins p8 into the growing phage (The model adapted from [5] with permission of Elsevier).

coat protein p8; and **c**) its spontaneous fusion with liposomes and micelles. We revealed that fusion coat proteins can accommodate several functional units [27] (**Fig.5).**

We found that capability of the selected landscape phage to specifically and selectively bind and penetrate into cancer cells is translated both to their individual proteins [29] and to cancer cell-targeted nanomedicines, inhibiting growth of cancer cells *in vitro* and *in vivo* [14, 21-28, 30-32].

The capacity of selected cancer cell-targeted phage proteins to navigate nanomedicines towards tumor cells *in vivo* was studied using subcutaneous and orthotopic **MCF-7** human breast cancer cell-induced tumor, (**Fig.7**). Micellar paclitaxel served as model drug-delivery vehicles. It was found that the effect of nanomedicine targeting on tumor growth depends not only on affinity, selectivity and specificity of *in vitro* selected phages towards corresponding cancer cells, but also on '*promiscuity*' of fusion phage

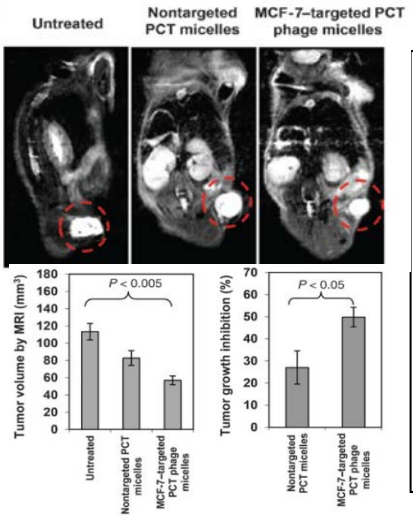

Fig.6 MRI images of tumors untreated and treated with nontargeted and MCF-7–targeted PCT phage micelles at the endpoint of treatment. Tumor growth inhibition (%) estimated by MRI, defined as the difference between the tumor volume of the untreated group and the tumor volume of the treated group divided by the tumor volume in untreated group × 100. Mean ± SEM, n= 5 * p<0.05 or p<0.005

proteins towards components of tumor microenvironment. For example, it was found that tumor-specific phage fusion coat protein **DMPGTVLP,** selected against human breast cancer cells MCF-7 [29] (phages and their fusion proteins are designated by the sequence of fused peptides) enhances binding and killing of target tumor cells by drug-loaded micelles in culture [24, 26, 28, 30] and in mice model [22, 23] (**Fig.6**). We explained the high efficacy of phage **DMPGTVLP** by its *promiscuity* [18]. We found [29] that octamer **DMPGTVLP** fused to **pVIII** phage protein, binds **nucleolin**—a phosphoprotein, which shuttles between the nucleus and the cytoplasm [33]. Nucleolin is overexpressed in cancer cells [33], and serves as a tumor angiogenic marker and biomarker for cancer diagnosis [34, 35]. The promiscuity of the DMPGTVLP fusion protein may expand to various cells of tumor microenvironment, such as angiogenic endothelial cells within the tumor vasculature, and others [35]. We hypothesized that the promiscuity of the phage DMPGTVLP is translated to the targeted nanomedicines,

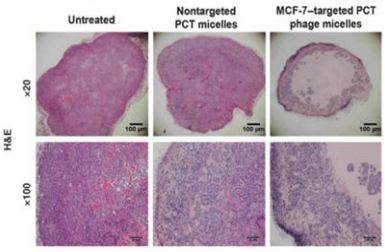

Fig.7 Representative images of hematoxylin & eosin staining of tumor sections. Viable cells show eosinophilic cytosol (pink) accompanied by hemato-xylin stained nuclei (blue) staining.

allowing them to migrate from blood stream to the tumor [36, 37], induce apoptosis of cancer cells and tumor reduction (**Fig.6,7**) [22, 23].

In sum, to improve the targeted therapy of breast cancer, we exploited the membranophilic MCF-7 cancer cell–specific phage protein to prepare tumor-targeted *P-micelles*, The targeted phage micelles produced a dramatic tumor reduction as well as extensive apoptosis and necrosis, as a result of the improved tumor delivery of paclitaxel. These results are indicative of translational potential of Landscape phage technology for improved breast cancer treatment

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