Landscape Phage Probes for PC3 Prostate Carcinoma cells

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ABSTRACT

Cancer chemotherapy is complicated by a lack of selective drug targets. However, tumor chemotoxicity of drugs can be enhanced by precisely targeting them to tumor receptors using tumor-specific probes. Peptide phage display offers a convenient approach to screen for tumor selective probes. Herein, we describe the use of landscape phage libraries for deriving specific phage probes for PC3 prostate tumor cells. Phage probes bound to the surface were collected by elution with acid buffer (Eluate fraction) whereas the phage probes internalizing into the cells were collected following the lysis of cells (Lysate fraction). Selected clones were tested for their target binding efficiency in cell association assays. The probes identified by these protocols will be studied as potential targeting ligands for liposomes and other nano-particulate drug vehicles.

Keywords: Phage display, Landscape phage, tumor-specific ligands

1 INTRODUCTION

Cancer diagnosis and treatment has probably posed one of the most serious challenges to modern medical research with the gravity of threat stemming from the provenance of the pathological tissue. The molecular propinquity of diseased and non-diseased tissues roils anti-cancer therapeutics leading to very serious side-effects. Although, anatomical aberrations like Enhanced permeability and retention [1] and over-expression of certain receptors like HER 2 [2] in tumors have been exploited to develop treatments selectively accumulating in malignancies such interventions fail to provide a molecularly directed therapeutic. Thus, alternative approaches for improving the therapeutic indices of available drugs have to be pursued. One such approach is to target anti-tumor drugs to the tumor sites thereby preventing their uptake and activity in non-malignant sites.

A plethora of ligands have been investigated for their capacity to deliver cytotoxic drugs or drug vehicles with each ligand having its attendant advantages and drawbacks. Peptides as ligands have the advantage of being small and therefore less immunogenic as well as more amenable to synthetic procedures. Furthermore, incorporation of D-amino acids additionally mitigates the immune response as well as ligand degradation by host enzymes. Given the size, peptides have ample opportunity of easily diffusing through the tumor mass as well as cancer cells in adjacent tissues. An additional advantage is the availability of peptide phage display libraries which allows for a rapid detection of target specific peptides. A general review on the applications of phage display libraries in cancer biology as well as approaches to harness phage display technology for personalized anti-cancer therapeutic production has been discussed by Samoylova et al [3]. Within this purview, a unique concept of landscape phage display has developed in which a phage manifests the foreign peptide on all copies of the major coat protein pVIII. The close knit arrangement of the multiple copies of the peptide transforms the phage surface creating novel landscapes to be used in interaction with an almost inexhaustible range of targets. Furthermore, the panoply of peptides allows for multivalent interactions creating the avidity effect. Cell-specific phage probes can be developed for unknown and non-immunogenic markers, are robust, stable on storage and easily obtainable by established microbiological procedures.

We describe here selection of landscape phage probes for PC3 prostate carcinoma cells using a biased selection scheme in which the pristine library is depleted of non-specific binders by exposure to auxiliary targets prior to exposure to the target cells. Selected phage clones after four rounds of selection were analyzed for their affinities towards the target cells using cell association assays. Based on our results we have identified clones which demonstrate 10-60 fold higher affinity for target cells as compared to serum.

2 LANDSCAPE LIBRARIES AS A SOURCE OF CANCER SPECIFIC LIGANDS

Peptide phage libraries evolved from a synergy of two concepts, combinatorial peptide libraries and fusion phage and are multibillion clone compositions of self-amplifying and self-assembling biological particles. The vehicles for the random peptides in the phage display system are the filamentous bacteriophages of the class Ff consisting of a single-stranded circular DNA packed in a cylindrical shell composed mainly of the major coat protein pVIII (98% of total protein mass) with small numbers of minor coat proteins at the ends of the phage particle. Landscape phages display the foreign peptide on every pVIII subunit, accomplished by an inframe splicing of degenerate synthetic oligonucleotides into the pVIII gene,

thereby increasing the virion's total mass by upto 15%. The intensive array of guest peptides dramatically alters the surface architecture of the phage particle creating novel milieu to serve as a scaffold for multivalent interactions with a diverse range of targets (see Fig 1). Thus, landscape phage represents a singular nanomaterial that can be selected in the affinity binding protocol and obtained by a routine and simple microbiological procedure. Binding peptides may be easily prepared *en masse* by propagating and purifying phage according to established protocols [4]. Landscape phages have been demonstrated to serve as substitutes for antibodies [5], diagnostic probes for bacteria and spores [6, 7], gene delivery systems [8], and biospecific adsorbents [9]. Phage derived probes inherit the robustness of wild-type phage [10] and can be utilized for fabrication of bioselective materials [11] or exploited as molecular recognition interfaces in detection systems [12]. Phage display libraries have been utilized to derive target-specific peptides for hepatocarcinoma [13], mouse melanoma [14] and lung tumors [15] to name a few. It was shown earlier that the tumor-specific peptides fused to the major coat protein pVIII can be affinity selected from multibillion clone landscape phage libraries by their ability to bind very specifically to cancer cells [16, 17] demonstrating a high potential of landscape phage libraries as a source of tumor specific ligands.

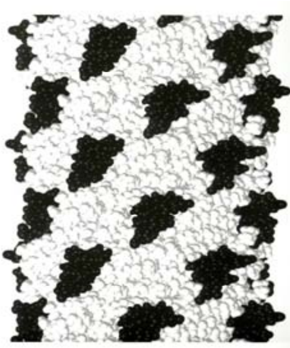

Figure 1: Structure of landscape phage. Foreign peptides are pictured with dark atoms; their overall arrangement corresponds to the model of Marvin, 1994.

3 PC3 PROSTATE CARCINOMA SPECIFIC PHAGE PROBES

3.1 Selection of PC3 cell specific phage peptides from landscape libraries

Two landscape phage libraries, one bearing octapeptide insert (f8/8) and the other bearing nonapeptide insert (f8/9) developed in our laboratory were used. In keeping with a long term goal of *in vivo* studies, we chose the PC3 cell line which is amenable to the development of a mouse xenograft model. For selection of tumor-specific peptides we adopted the selection protocol of Samoylova et al [17] with some essential changes. In an effort to minimize the selection of peptides binding non-specifically and maximize the affinity of target binding peptides we implemented a rigorous library depletion regimen wherein the library was consecutively exposed to culture flasks, serum treated culture flasks as well as non-target cells (fibroblasts) before being incubated with PC3 cells. Following extensive washing of the cells to remove unbound phage, phage bound to the surface were collected by elution with acid buffer (pH 2.1). After further washing, cell internalized phage particles were obtained by lysing the cells in sodium deoxycholate. Each phage fraction was amplified and used as input library in subsequent rounds of selection. The ratio of cell associated phage (eluate and lysate fraction) to the input phage termed as the yield was monitored for each round of selection (see Fig 2). An increase in this parameter for both fractions across successive rounds of selection implies a progressive selective enrichment of the phage library for target specific clones. After four rounds of selection, selected phage clones were isolated and sequenced (see Table 1).

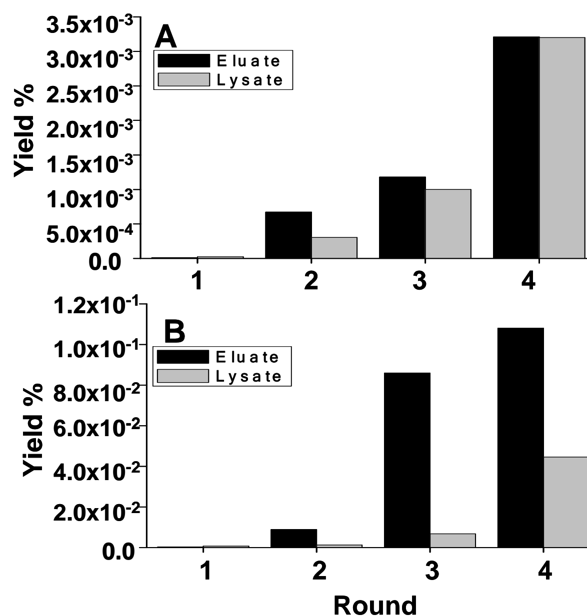

Figure 2: Phage output during successive rounds of selection. A – f8/8 library. B – f8/9 library.
X axis – round of selection, Y axis – ratio of phage output to input expressed as percentage. A progressive increase in the yield across successive rounds indicates selective enrichment of library for target specific clones.

Octapeptide insert library		Nonapeptide insert library	
Eluate	Lysate	Eluate	Lysate
DTDSHVNL	DLTYVNSQ	AAYDVNVND	DVVYALSDD
GDNSHVNL	DSSNKPTG	AEYGESVNA	EAAGANIAP
GPDSTWAG	DSSRLERV	DSDVGWVND	
VSDNTDYS	ERAPLSVE	EAAGANIAP	
DTPYDLTG		VDVSEQMSL	
		VGDYDVVDS	

Table 1: Representative peptide structures of selected clones obtained after sequencing.

NSTI-Nanotech 2008, www.nsti.org, ISBN 978-1-4200-8504-4 Vol. 2

3.2 Target specificity of selected clones

Individual phage clones identified by sequencing were propagated and purified to be used in specificity assays. In these assays, the association of a selected clone with target PC3 cells as opposed to serum was monitored. The protocol essentially followed that of selection with a pre-determined titer of phage particles being incubated with target as well as serum treated control wells of a 24-well cell culture plate. Following washing, cell or serum associated phage was collected by lysing the cells and titered. Figure 3 demonstrates the results of these assays. Clone DTDSHVNL from the octapeptide insert library demonstrated a nearly 10 fold better affinity towards cells than towards serum whereas clone GAYDVNVND from the nonapeptide insert library demonstrated nearly 60 fold better affinity towards target cells as compared to serum. Phage bearing unrelated peptide, VPEGAFSS and vector phage bearing no peptide were used as controls and demonstrated negligible binding to cells.

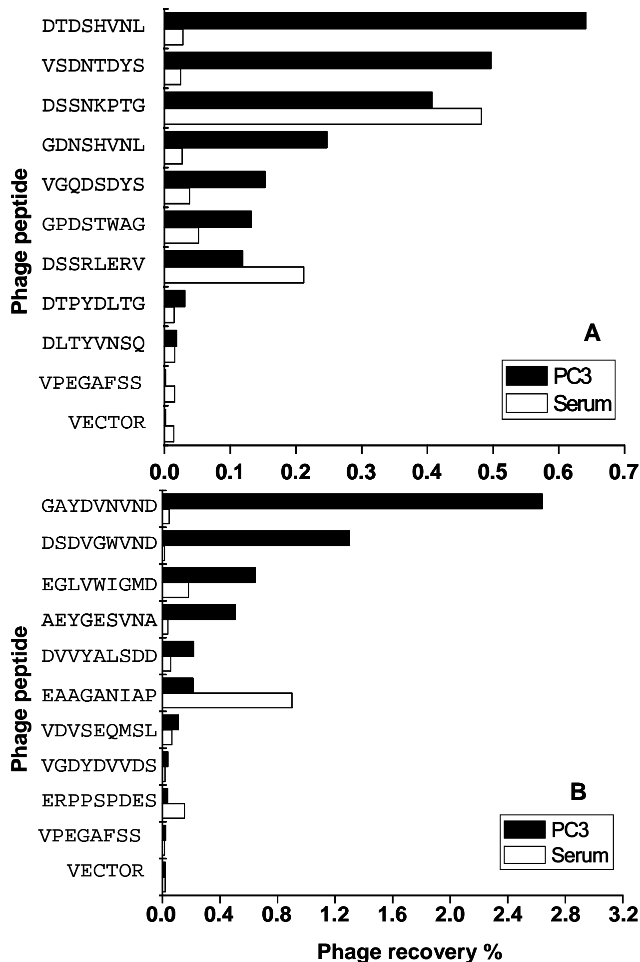

Figure 3: Cell specificity of selected phage peptides. A – f8/8 library. B – f8/9 library. Selected phage clones were tested for their affinity towards PC3 cells as compared to serum.
Y – Axis: ratio of phage recovered from cells or serum to input phage expressed as percentage

4 CONCLUSION

Undoubtedly, phage display technology permits a high throughput system for discovery of tumor-specific peptides with the identified peptides being harnessed as putative targeting ligands for drugs/drug vehicles/imaging agents [18-21]. The development of *in vivo* selection techniques has further provided an impetus in the broad spectrum application of phage display libraries [22, 23]. Peptides as targeting moieties possess considerable potential owing largely to their small size and successful use of peptides as targeting ligands has been demonstrated hitherto [24-29]. A variety of techniques for coupling peptides to carrier molecules have also been developed [30]. These techniques, though efficient for conceptual experiments are prohibitive in large scale applications, creating a bottle neck in the development of targeted drugs. Within this context, the unique structural and biochemical properties of phage major coat protein merits further attention. The phage major coat protein is an intrinsic membrane protein with a propensity to insert into lipid bilayers when separated from the phage DNA. The amphiphilic nature of the protein drives it to incorporate into liposomes where it forms a transmembrane moiety firmly anchored in the bilayer with the N-terminus exposed on the surface of the bilayer [31]. We propose to harness this property to incorporate tumor specific peptides into therapeutic liposomes thereby deriving tumor targeted liposomes (see Fig 4). Earlier research in our laboratory had demonstrated the intrinsic capability of isolated target specific fusion phage proteins to assemble into lipid bilayers and instill an emergent property in the liposomes, that of target selectivity [32]. The simplicity of the approach coupled with the ability to rapidly obtain tumor specific phage fusion proteins via phage display allows one to envisage a combinatorial system for the production of targeted liposomal therapeutics.

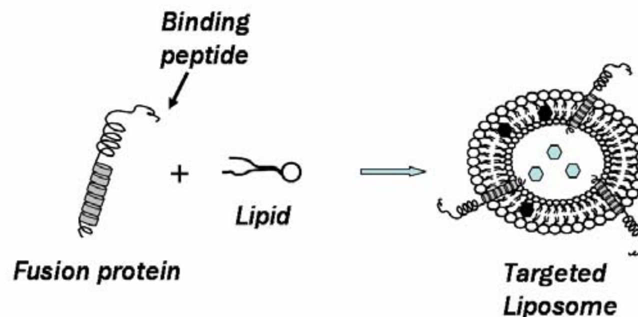

Figure 4: Drug-loaded liposome targeted by the tumor specific pVIII protein. The hydrophobic helix of pVIII spans the lipid layer and tumor specific binding peptide is displayed on the surface of the carrier particles. Drug molecules are displayed as hexagons.

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