Targeted Drug-carrying Filamentous Phage Nanomedicines

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ABSTRACT

We present a novel application of filamentous bacteriophages as targeted drug carrying nanomedicines. The phages are engineered to display target-specificity-conferring peptides or proteins on their coat, and carry a large payload of a cytotoxic drug that is conjugated to the phage major coat protein via a labile linker subject to controlled or delayed release. We show growth inhibition of target bacteria or cancer cells with impressive potentiation factors over the corresponding free drugs. We further show that targeted drug-carrying phages are non-toxic to mice and that their immunogenicity is reduced as a result of drug conjugation. Our approach replaces the selectivity of the drug itself with target selectivity born by the targeting moiety, which may allow the use and re-introduction of "non-specific" drugs that have thus far been excluded from antibacterial use (due to toxicity or low selectivity).

Keywords: Antibiotic resistance; Immunogenicity; Phage nanomedicines, Targeted immunotherapy.

1 INTRODUCTION

Since the introduction of monoclonal antibodies (mAbs), and the initial clinical trials of antibody therapy in cancer patients, there has been progress in antibody based therapeutics. In general, drug immunoconjugates are composed of targeting entities (mainly mAbs) chemically conjugated to a cytotoxic drug. The outcome is improved drug efficacy with reduced systemic toxicity. To date, the most clinically-advanced forms of armed antibodies are antibody-isotope and antibody-drug conjugates. Key issues in designing and testing immunoconjugates include: 1. the nature of the target molecule, its abundance at the target, whether it is internalizing and at what rate, and its specificity to the target, cells or tissues. 2. the linkers used to attach the drug to the targeting moiety. 3. the drug carrying capacity of the carrier is also a key issue in its potency.

A second class of targeted drug delivery platforms are the drug-carrying nanomedicines, such as liposomes, nanoparticles, drug-loaded polymers and dendrimers. Most nanomedicines do not utilize a targeting moiety to gain target specificity. Rather, they rely on the "enhanced permeability and retention" (EPR) effect that results from the rapid deployment of blood vessels within rapidly growing tumors resulting in blood vessels in the tumor being irregular in shape, dilated, leaky or defective. As a result, large drug-carrying platforms may gain selective access to the tumor while their exit at non-target sites is limited. While the immunoconjugates are limited in drug-carrying capacity, usually less than 10 drug molecules per targeting moiety, nanomedicines by nature deliver a much larger payload to the target cells.

Filamentous bacteriophages (phages) are the workhorse of antibody engineering and are gaining increasing importance in nanobiotechnology. Phage-mediated gene delivery into mammalian cells was developed following studies that identified “internalizing phages” from libraries of phage-displayed antibodies or peptides. Recently, an efficient integrated phage/virus system was developed by the Pasqualini-Arap group where tumor targeting and molecular-genetic imaging were merged into an integrated platform [1].

We have been studying the potential of phages to serve for targeted delivery by applying them as anti bacterial and anti tumor cells nanomedicines. The phages were genetically engineered to display a target-cell specificity-conferring molecule on all copies of the phage g3p minor coat protein. The targeted phages were chemically conjugated, via a cleavable bond to a large payload of a drug, with a maximal loading capacity of more than 10,000 drug molecules/phage. We present the results of growth inhibition studies and of toxicity and immunogenicity studies mouse studies that underscore the potential of targeted drug-carrying nanomedicines for further development. The concept is schematically illustrated in Figure 1.

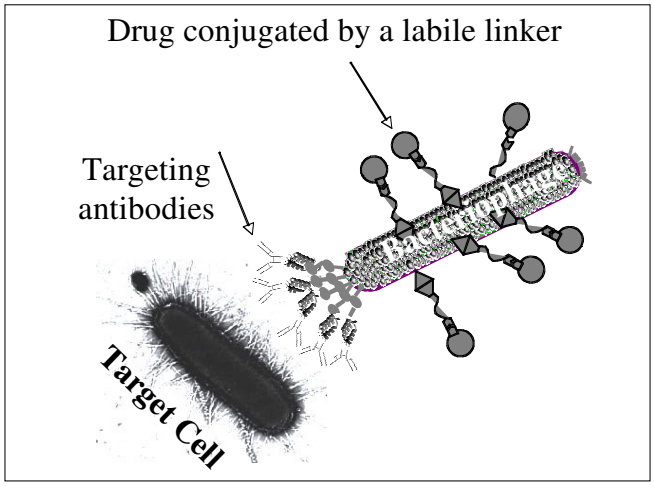

Figure 1. Scheme of the targeted drug-carrying phage nanomedicines system.

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2 RESULTS

2.1 Anti bacterial studies

While the resistance of bacteria to traditional antibiotics is a major public health concern, the use of extremely potent antibacterial agents is limited by their lack of selectivity. As in cancer therapy, anti bacterial targeted therapy could provide an opportunity to re-introduce toxic substances to the anti-bacterial arsenal. A desirable targeted anti-bacterial agent should combine binding specificity, a large drug payload per binding event and a programmed drug release mechanism.

We present a novel application of filamentous bacteriophages as targeted drug carriers that could inhibit the growth of target bacteria. Conjugation of the model drug, chloramphenicol, to phages was carried our through aminoglycoside linkers. This was a solution to the limited arming efficiency that was mainly due to drug hydrophobicity that we observed in our initial studies [2] The application of highly water-soluble aminoglycoside antibiotics as branched solubility-enhancing linkers overcame the hydrophobic barrier in aqueous solutions on one hand, and significantly increased the loading potential of drug molecules per phage particle on the other. The drug was conjugated to carboxyl residues on the phage coat using EDC chemistry. This resulted in drug carrying capacity potential of >10000 molecules per phage.

The target bacteria we used as models were *Staphylococcus aureus*, *Streptococcus pyogenes* and an avian pathogenic *E. coli*O78 strain. The phages displayed an IgG binding ZZ domain on the g3p minor coat protein, allowing efficient complex formation with targeting antibodies. We complexed the phages with polyclonal antibodies that served as the targeting moieties in our targeted drug-carrying platform.

The growth inhibition experiments with staphylococci were done with the minimal amount of phages that gave total growth inhibition, 10^{10} phage particles per 10^7 bacteria. Negative controls were non treated bacteria as well as bacteria treated with non immune human IgG or human Fc complexed bacteriophages conjugated to the same amount of antibiotic. The results were compared to growth inhibition resulting from varying concentrations of free chloramphenicol. We found that 10^{10} targeted drug-carrying phages inhibited bacterial growth as do 15 µg of free chloramphenicol (Figure 2). Similar growth inhibition experiments were carried out with the other bacterial targets where we could also observe growth inhibition. The growth inhibition profile of *Streptococcus pyogenes* and *E. coli* O78 by free chloramphenicol was similar to that of the *Staphylococcus aureus* bacteria.

We calculated the potency improvement factor by assuming that the fraction of relevant phages (that bind the target bacteria) is equal to the fraction of target-relevant IgGs within the sera. Based on that 10^{10} targeted

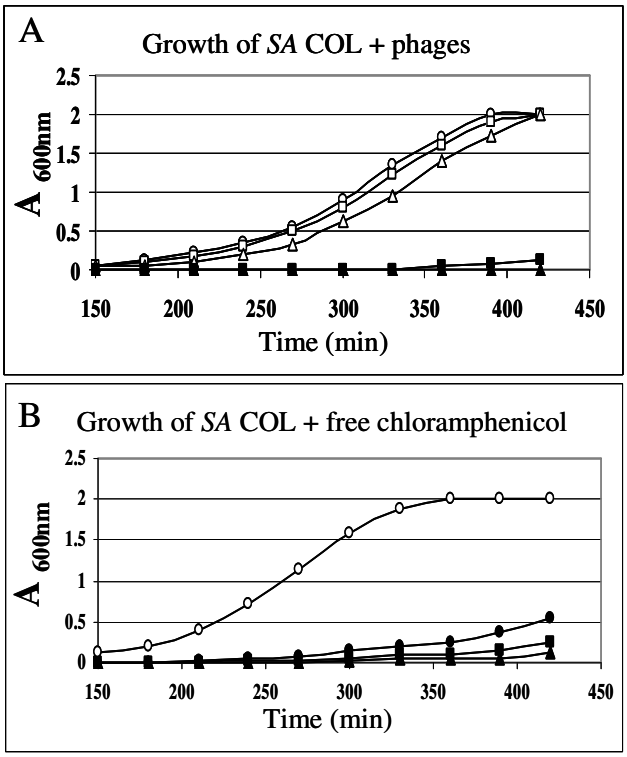

Figure 2. Inhibition of bacterial growth by targeted antibiotic-carrying phages: growth inhibition curves. A. Growth of *Staphylococcus aureus* treated with 10^{11} (▲) or 10^{10} (■) of targeted drug-carrying phages. Controls are cells treated with or 10^{11} (Δ) or 10^{10} (□) of drug-carrying phages conjugated to human Fc, and cells treated with targeted fUSE5-ZZ phages that do not carry drug (O). B. Growth of *SA* in the presence of varying concentrations of free chloramphenicol: 20 µg (▲); 15 µg (■); 10 µg (●) and untreated (O).

chloramphenicol-carrying phages inhibiting bacterial growth as effectively as did 15 microgram of free chloramphenicol. Considering that, based on the fraction of target-specific IgG in the serum was about 5% of the phages are targeted, which for 10^7 bacteria yields 50 drug-carrying phages that actually bind each target bacterium. Each phage carries ~10^4 chloramphenicol molecules, of which 30% (3000) are released during the time course of the experiment (based on release kinetics reported in [1]) This yields 150,000 drug molecules released for each target bacterium. For 10^7 target bacteria, 15 µg of free chloramphenicol correspond to ~3×10^9 molecules/bacterium. Hence, the potency improvement factor in comparison to the free drug is about 20,000 [3].

2.2 Anti cancer studies

As target cells we used several cell lines that express varying levels of ErbB2 or EGFR on their surface. Antibodies were complexed to drug-carrying phages through the ZZ domain displayed on g3p. We found that the

antibody-complexed phages exhibited cell specific binding which corresponded to the level of antigen expression on the target cells. Next, evaluation of phage internalization into target cells was carried out using confocal microscopy. We found that targeted drug carrying phages could internalize into SKBR3 and also into A431 target cells.

We evaluated two model drugs that were conjugated to the phage coat using EDC chemistry, forming an amide bond between the exposed carboxyl side chains on the phage coat, mostly the ones exposed on g8p, and a free primary amine on the drugs. The drugs we used were hygromycin (an aminoglycoside antibiotics) or doxorubicin (an anthracycline antibiotic). Approximately 10000 molecules of hygromycin were conjugated to each phage. In this example, EDC reaction caused the formation of a covalent bond between the phage major coat protein, g8p and the drug which does not facilitates a controlled release form of the drug at the target site. However, as shown in Fig. 2, the targeted phage nanoparticles are internalized into the cells possibly entering the lysosomal compartment where they are susceptible to digestion by lysosomal proteases. This led us to the assumption that lysosomal deconstruction of the phage may mediate drug release within the cell.

To obtain controlled release of the conjugated drug, fUSE5-ZZ-(g8p)DFK phage was designed. fUSE5-ZZ-(g8p)DFK phages display the lysosomal cysteine protease cathepsin-B cleavage site on the phage major coat protein, g8p. Following doxorubicin conjugation to the phages we evaluated drug release mediated by cathepsin-B. As shown in Figur 3, drug release could be observed by the red color of the phage-free supernatant following PEG/NaCl precipitation of cathepsin-B-treated, doxorubicin conjugated fUSE5-ZZ(g8p)DFK phages. MALDI-MS analysis of the released material identified it as an Asp-doxorubicin adduct that was released owing to the genetic-engineered release mechanism.

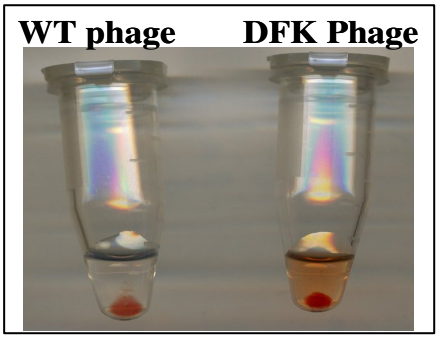

Figure 3. A Photograph of the *in vitro* cathepsin-B release experiment tubes. On the right, doxorubicin carrying fUSE5-ZZ-(p8)DFK phages that was incubated with cathepsin-B, followed by PEG/NaCl precipitation, a reddish soluble D-DOX can be seen. On the left is a tube containing doxorubicin conjugated fUSE5-ZZ phages that was incubated with cathepsin-B, followed by PEG/NaCl precipitation, the transparent colorless solution indicate no drug release.

Evaluation of the cell cytotoxicity of hygromycin carrying fUSE5-ZZ phages complexed with an anti ErbB2 antibody, chFRP5 was done by MTT assays. ErbB2-expressing SKBR3 cells were incubated for 48 h with 5×10^{11} of hygromycin carrying phages, and the relative number of viable cells in comparison with cells grown in the absence of the phage was determined. We could show that hygromycin carrying fUSE5-ZZ-chFRP5 phages inhibited target cell growth by 50%, (Figure 4), a >1000 fold improvement in hygromycin potency (in comparison to the free drug). No killing was observed when the cells were treated with hygromycin conjugated fUSE5-ZZ-phages in complex with human IgG (non-targeted) or to targeted fUSE5-ZZ-chFRP5 that were not conjugated to the drug. In contrast, the viability of HEK293 cells that were treated with the same dose of hygromycin carrying phages was not affected at all.

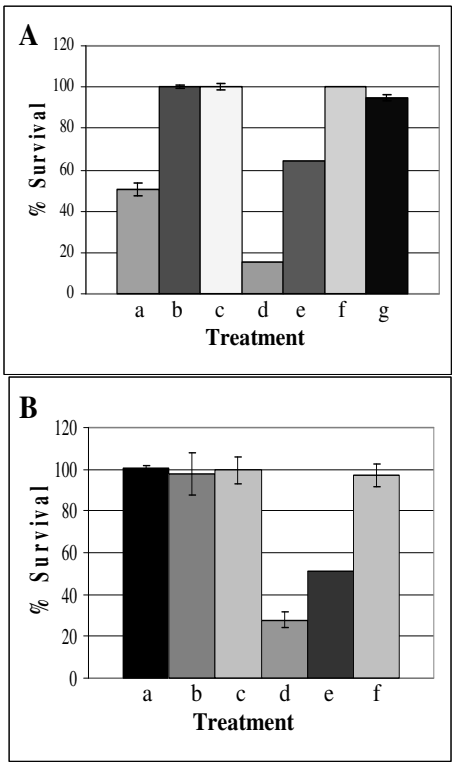

Figure 4. Growth inhibition cultured cells by hygromycin carrying phages. **A.** SKBR3 (target) cells were incubated with 5×10^{11} hygromycin carrying fUSE5-ZZ–chFRP5 phages (a), hygromycin carrying fUSE5-ZZ–human IgG (b), fUSE5-ZZ–chFRP5 phages (no drug) (c), 2 mg free hygromycin/well (d), 0.2 mg free hygromycin/well (e), 0.02 mg free hygromycin/well (f) 0.002 mg free hygromycin/well (g). **B.** Hygromycin carrying phages on HEK293 (non-target) cells. Cells were incubated with 5×10^{11} of hygromycin carrying fUSE5-ZZ–human IgG phages (a), hygromycin carrying fUSE5-ZZ– trastuzumab phages (b), hygromycin carrying fUSE5-ZZ– cetuximab phages (c), 1 mg/ml free hygromycin (d), 0.1 mg/ml free hygromycin (e), 0.01 mg/ml free hygromycin (f).

Evaluation of the cell cytotoxicity of doxorubicin conjugated fUSE5-ZZ(p8)DFK in complex with the anti ErbB2 antibodies trastuzumab (Herceptin®) or anti EGFR cetuximab (Erbitux®) was done using MTT assays with A431 and SKBR3 cells. Here the drug was designed to be released in a controlled manner. Doxorubicin was conjugated to the phage using the EDC chemistry, through the engineered DFK tri-peptide where the drugs release is mediated through the cathepsin-B activity in the endosomal-lysosomal compartments. We found that doxorubicin carrying fUSE5-ZZ(g8p)DFK in complex with each of the targeting IgGs, but also with the non-targeting human IgG caused efficient killing of the target cells, in a dose-dependent manner. When doxorubicin was conjugated to fUSE5-ZZ phages in complex with trastuzumab (without the DFK sequence), growth inhibition was minimal.

2.3 Mouse studies

To evaluate toxicity and immunogenicity of drug-carrying phages to mice, we injected several doses IV or IP, at 2 week intervals for a total of three injections. All the mice tolerated the treatment well up to the highest injected dose which was 10^{11} phages / injection.

To evaluate immunogenicity, mice were bled 2 weeks after each injection, and also 3 and 6 months from initiation of the experiment. We found that native (not conjugated) phages, and doxorubicin-carrying phages were highly immunogenic. In contrast, phages that were conjugated to chloramphenicol via an aminoglycoside linker had a markedly reduced immunogenicity (Figure 5).

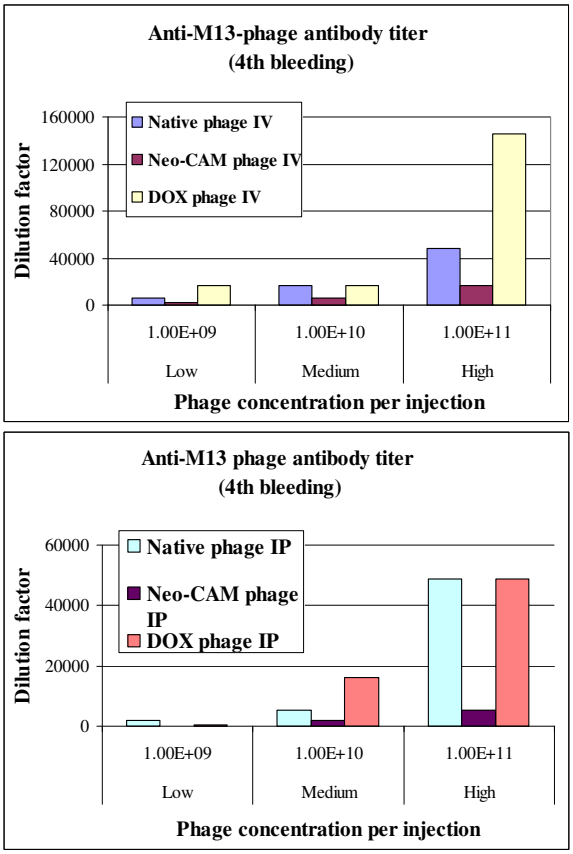

Figure 5. Measuring phage immunogenicity. ELISA plates were coated with 10^{11} fUSE5-ZZ phages /well. Reactivity of sera were determined by incubating serial dilutions of the sera in the phage coated well and detection of the bound antibodies using HRP-conjugated rabbit anti mouse antibodies. This particular experiment shown the bleed taken 3 months after the initial injection. Top panel: IV injections, bottom panel, IP injections.

A preliminary pharmacokinetic study showed that phages that were conjugated to chloramphenicol via an aminoglycoside had a prolonged circulation time in comparison to native phages (Figure 6).

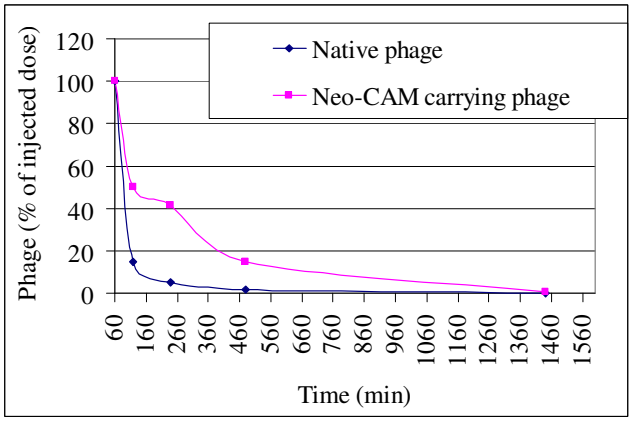

Figure 6. Measuring blood pharmacokinetics. Mice were injected with 1011 phage particles IV and bled at varying time point post injection. Phage titer was determined by titration of sera dilutions on infection-susceptible *E. coli* cells for determination of phage CFU.

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