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Original Article

Discovery of peptide drug carrier candidates for targeted multi-drug delivery into prostate cancer cells

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ABSTRACT

Metastatic castration-resistant prostate cancer (mCRPC) remains essentially incurable. Targeted Drug Delivery (TDD) systems may overcome the limitations of current mCRPC therapies. We describe the use of strict criteria to isolate novel prostate cancer cell targeting peptides that specifically deliver drugs into target cells. Phage from a libraries displaying 7mer peptides were exposed to PC-3 cells and only internalized phage were recovered. The ability of these phage to internalize into other prostate cancer cells (LNCaP, DU-145) was validated. The displayed peptides of selected phage clones were synthesized and their specificity for target cells was validated in vitro and in vivo. One peptide (P12) which specifically targeted PC-3 tumors in vivo was incorporated into mono-drug (Chlorambucil, Combretastatin or Camptothecin) and dual-drug (Chlorambucil/Combretastatin or Chlorambucil/Camptothecin) PDCs and the cytotoxic efficacy of these conjugates for target cells was tested. Conjugation of P12 into dual-drug PDCs allowed discovery of new drug combinations with synergistic effects. The use of strict selection criteria can lead to discovery of novel peptides for use as drug carriers for TDD. PDCs represent an effective alternative to current modes of free drug chemotherapy for prostate cancer.

Introduction

Metastatic castration-resistant prostate cancer (mCRPC) is essentially incurable [1] and in 2014 the disease accounted for approximately 30,000 deaths in the US alone [2]. Androgen deprivation therapy, for many years the standard first-line treatment for mCRPC is initially effective in most patients, but disease inevitably progresses [3]. Newer molecular targeted therapies have shown clinical benefit for patients with mCRPC including chemotherapies, immunotherapies and radiopharmaceuticals. However it is not yet clear how to sequence these therapies in individual patients to achieve long-term clinical efficacy with low toxicity, nor is it clearly understood how cross-resistance to each component develops (reviewed in Ref. [3]. The continual genetic evolution of prostate cancer (PrC) cells is an additional complication [4], requiring repeated patient monitoring to update chemotherapy regimens [3], a process of “cat-and-mouse” that is expensive and carries implications for drug toxicity, patient morbidity and survival.

Targeted Drug Delivery (TDD) systems based for example on antibodies [5] and peptides [6]may overcome many of the limitations of current mCRPC therapies. For example our own studies demonstrate that attachment of a small ligand- or peptide-cancer cell targeting agent to cytotoxic drugs affords several advantages that include: specific delivery of drug to the target cell, regulated cytotoxicity, bypassing of drug resistance, reversal of drug resistance, delivery of higher drug payloads and simultaneous delivery of cytotoxic drugs with different mechanisms of action [7–12].

TDDs assume the presence of an appropriate cell surface target molecule and one of the most studied mCRPC related cell surface components is Prostate Specific Membrane Antigen (PSMA), a 100 KDa type II transmembrane glycosylated protein. PSMA is very commonly expressed on PrC cells. The biology and clinical relevance of PMSA expression has been extensively researched and it has been targeted by a diverse selection of small ligands, antibodies and peptides to which have been conjugated a variety of radiolabels

Abbreviations: (mCRPC), Metastatic castration-resistant prostate cancer; (TDD), targeted Drug Delivery; (PDC), Peptide-Drug-Conjugate; (PrC), Prostate cancer; (PSMA), Prostate Specific Membrane Antigen; (GnRH), Gonadotropin-Releasing Hormone; (Chlor), Chlorambucil; (Cpt), Camptothecin; (Comb), Combretastatin.

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and drugs [13,14]. Some of these constructs have been evaluated clinically (some are still ongoing, see www.clinicaltrials.gov) and FDA-approved PSMA-targeted products are available for diagnostic imaging [15]. Despite these advances, many questions remain about the efficacy of PSMA-targeted therapeutics [13–15].

The most clinically advanced peptide-based TDD systems are bioconjugates incorporating analogues of the natural hormone Gonadotropin-Releasing Hormone (GnRH) (reviewed in Ref. [16]) that target the GnRH receptor. However, this approach has several limitations. a) GnRH receptors are also present on a variety of normal tissues. b) Due to the structure of the receptors, internalization of the bioconjugate is relatively slow; c) It has proven very difficult to prepare active conjugates of these analogues with drugs other that anthracyclines such as doxorubicin [16].

To expand the spectrum of targeting peptides for TDD, phage display technology is being used [17,18]. Jayanna et al. [19] and also Fagbohun et al. [20] used phage display to isolate clones that were internalized by PrC cells, with the aim of using the phage themselves, not the isolated peptides, as delivery vehicles. The Arap group [21]recently described phage clones internalized by prostate cancer cells in bone metastases, however activity with isolated peptides was not described. Other studies focused on using phage libraries to isolate peptides binding other known receptors on PrC cells [22–25]. However, the peptides were mainly designed to inhibit the activity of a receptor.

Our goal was different. We set out to use phage display peptide libraries to isolate novel PrC targeting peptides for use in Peptide-Drug-Conjugates (PDCs) that would be effective in the treatment of mCRPC. Our approach was to use unbiased exposure of the phage library to mCRPC cells, without selecting for peptides that bound a pre-defined receptor. We used strict criteria to select appropriate phage clones and their displayed peptides: a) internalization of the phage into the target cell, b) target cell specificity c) uptake of the synthesized display peptide into target cells d) accumulation of the peptide into tumor and e) incorporation of the peptide into both mono-drug and dual-drug-PDCs that killed PrC cells.

We compared a commercial phage library expressing 7-mer linear peptides with the same library after it had been injected into a normal mouse. Phage clones recovered from the bloodstream of the mouse were assumed not to have effective affinity for normal tissues and were not degraded by the liver. This manipulation significantly reduced the number of clones in the library, thus increasing the probability of “fishing out” appropriate on-target clones. Indeed the two peptides (P10 and P12) that we have so far tested most thoroughly and are reporting on here were both derived from the second library. These peptides are active on a range of metastatic prostate cancer cell lines but not on normal cells. Importantly, the preparation of active peptide-dye conjugates was found to be terminus dependent and this guided the synthesis of PDCs that were cytotoxic to PrC target cells. These peptides are now moving into an advanced program of pre-clinical testing as a potential novel targeted drug therapy for mCRPC.

Material and methods

Cells and reagents

The cell lines used in this study represented malignant prostate cancer (PC-3, LNCaP, DU-145), cervical cancer (HeLa) and human embryonic kidney cells (HEK-293). All cells were obtained from the ATCC. PC-3, DU-145 and LNCaP were grown in RPMI 1640 (Thermo Fischer Scientific, Waltham, Ma., USA). HeLa were grown in MEM Alpha medium and HEK-293 in DMEM (Thermo Fischer). All media were supplemented with 10% FBS (Thermo Fischer), 3 mM L-Glutamine and antibiotics (Biological Industries, Bet Haemek, Israel). Cells were maintained at 37 °C and 5% CO_2 and retrieved using a rubber policeman. Normal lymphocytes were obtained from a Balb/c mouse spleen.

The phage display peptide library kit (Ph.D-7) was purchased from New England Biolabs (Ipswich, MA, USA). The library contains 10^{10} pfu/μl. This includes about 10^8 phage clones/μl, each displaying a unique linear 7mer linear peptide. The kit was used according to the manufacturer's instructions. Chemicals and reagents for peptide isolation and other procedures were purchased either from Sigma-Aldrich (Rehovot, Israel), Hylabs (Rehovot, Israel) or Bio-labs (Jerusalem, Israel). Rabbit polyclonal and mouse monoclonal antibodies to M13 + Fd coliphage were from Abcam. Peptides were synthesized by Pepmic CO. Ltd, Suzhou, China.

Selection of PrC internalized phage clones

To isolate peptides that might be used to specifically deliver cytotoxic drugs into prostate cancer cells, two phage display peptide libraries were used. The first (termed L1) was the parent Ph.D-7 library. The second library (L2) was derived from L1 and comprised phage that did not bind normal mouse tissues. For this purpose, 10^{11} pfu of L1 (10 μl) in 100 μl PBS were injected intravenously into a Balb/c mouse and phage were recovered from the blood 24 h later using the phage isolation procedure described in the kit. The in vivo absorption process resulted in a reduction of the phage concentration from 10^{10} pfu/μl to10^5 pfu/μl (data not shown). This absorbed phage pool is referred to as the L2.

PC-3 cells were seeded onto a well of a 96 well microplate (Nunc Maxisorb) for 24 h to bring the culture to about 75% confluence. The cells were washed with PBS and then exposed to 10^9 phage particles from either L1 or L2 libraries in 100 μl PBS. The microplate was placed on a planetary rotator for 1 h at room temperature. The non-absorbed phage were recovered, the cells were washed with PBS and then 100 μl of 0.2 M Glycine-HCl, pH. 2.2 were added to elute cell-bound phage. After 10 min, 15 μl of 1 M Tris-HCl, pH 9.1 was added to neutralize the solution and the eluted phage were recovered. The cells were washed again with PBS and then 100 μl 30 mM Tris, pH. 8.0 was added. The cells were frozen-thawed until they had ruptured as judged by light microscopy (three cycles). The ruptured cell mixture was collected and centrifuged at 2000 rpm for 2 min; the supernatant containing the PC3 internalized phage was recovered. Phage were titrated, amplified and the exposure procedure was repeated twice more. Phage from the last (third) round were titrated and those from 15 isolated, randomly selected plaques were individually recovered. The phage from each plaque were amplified, their DNA isolated and sequenced and the sequence of their peptide inserts deduced.

Comparing the capacity of isolated phage clones to internalize into PrC cell lines

To assess whether the PC-3 internalizing phage would also target other PrC cells, we adapted a procedure based on the classical ELISA calibration assay. Phage were diluted (10^{10}, 10^9, 10^8/μl) in PBS and incubated with prostate cancer cell lines (PC-3, LNCaP or DU-145) for 1 h. Cells were washed, lysed and internalized phage recovered and titrated as described above. Those clones giving the highest ratio between input and internalized phage for each input concentration were considered as having greater pan-PrC internalizing capacity.

Synthesis of Peptide-FITC and peptide-drug conjugates

The solid phase based synthesis of all peptides was performed by following a previously described procedure [10–12,26]. Preliminary results had shown that for both peptides, labeling at the C-terminus significantly impeded peptide labeling of the cells (data not shown). Therefore for all conjugates described here, labels and drugs were added to the N-terminus of the peptides. Complete details of the procedures and conditions, including those for the synthesis of peptide-FITC conjugates, synthesis of mono- and dual-drug PDCs, reverse phase HPLC purifications and HPLC/LC-MS analyses are detailed in Supplementary Data.

In vitro specificity of PrC internalizing peptides

To test the effect of conjugation on peptide activity, P10 and P12 were conjugated to FITC at either the N- or C- peptide terminus. The labeled peptides were then incubated with PC-3 at different concentrations and the fluorescence signal from cells was measured using a FACS Calibur flow cytometer (Becton Dickinson, USA) and analyzed by FlowJo software (FlowJo LLC, USA). Results showed that for both peptides, labeling at the C-terminus significantly impeded peptide labeling of the cells (data not shown). Therefore all subsequent experiments were performed with N-terminus labeled peptides.

To assess the range of binding activities of P10- and P12-FITC, labeled peptides were incubated at different concentrations (0–1 μM) with PC-3, DU-145, HeLa, HEK-293 cells and normal mouse splenic lymphocytes for 40 mins at room temperature. Cells were then washed and % of fluorescently labeled cells was measured by flow cytometry. A scrambled P12-FITC compound was also synthesized and compared to P12-FITC in a dose-response experiment for binding to PC-3 cells.

To visualize internalization of P12 into cells, the conjugates were incubated for 3 h at 37 °C with PC-3 cells. After incubation the cells were washed three times with PBS and DAPI was added. Internalized fluorescent signal was imaged with a Zeiss LSM700 confocal microscope.

In vivo tumor localization of PrC peptides

Preliminary tumor localization studies were performed. Male nude mice were inoculated subcutaneously with 2 × 10^6 PC-3 cells. Nineteen days later, when