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Design and evaluation of a peptide-based immunotoxin for breast cancer therapeutics

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ABSTRACT

Immunotoxins are chimeric proteins comprising a specific cellular targeting domain linked to a cytotoxic factor. Here we describe the design and use of a novel, peptide-based immunotoxin that can initiate selective cytotoxicity on ErbB2-positive cells. ErbB2 is a receptor tyrosine kinase that is overexpressed in the tumor cells of approximately 30% of breast cancer patients. Immunotoxin candidates were designed to incorporate a targeting ligand with affinity for ErbB2 along with a membrane lysin-based toxin domain. One particular peptide candidate, NL1.1-PSA, demonstrated selective cytotoxicity towards ErbB2-overexpressing cell lines. We utilized a bioengineering strategy to show that recombinant NL1.1-PSA immunotoxin expression by *Escherichia coli* also conferred selective cytotoxicity towards ErbB2-overexpressing cells. Our findings hold significant promise for the use of effective immunotoxins in cancer therapeutics.

1. Introduction

One of the biggest challenges facing cancer treatment is the development of resistance to chemotherapy [1]. In order to combat this treatment barrier, various attempts at specialized, targeted therapies are emerging. Among them are the concepts of gene therapy [2] and recruitment of the host immune system to fight against tumor formation and progression [3–5], although each approach is hampered with its own set of limitations. Importantly, many advancements are being made with small molecule inhibitors toward receptor tyrosine kinases that are often overexpressed in cancers, but the majority of current FDA-approved inhibitors are not targeted to a particular molecule, frequently resulting in strong side effects and premature drug resistance [6]. Therefore, the development of more specific targeted therapies is of critical importance. One such therapeutic approach that has begun to garner interest is the development of immunotoxins [7,8].

Immunotoxins are chimeric proteins containing a targeting moiety conjugated to a toxin component [8]. The targeting moiety is frequently an antibody or recombinant antibody fragment [9], a design that has recently shown promise in treating blood cancers such as leukemia [10].

The toxin component of immunotoxins can be synthesized from a variety of sources, including derivatives from plants or bacteria [7,8]. For years, attempts have been made to engineer live bacterial cells to target tumors and induce cell death [11,12]. However, due to immune rejection and additional adverse side effects, focus has shifted to using bacterial-derived agents for cancer treatment instead [11]. At present, the only immunotoxin approved by the FDA is denileukin diftitox, a protein immunotoxin consisting of interleukin 2 (IL-2) as the targeting ligand, and a truncated version of the Diphtheria bacterial toxin [13,14]. It is used for the treatment of recurrent cutaneous T-cell lymphomas [15]. Similar to the ADP-ribosylating action of Diphtheria toxin (which acts to arrest protein translation), another immunotoxin strategy has been developed using the protein secreted by *Pseudomonas aeruginosa*, *Pseudomonas* exotoxin A [16]. Recent conjugation of this toxin to melanocyte-stimulating hormone has proven highly effective in the treatment of melanoma in mice [17]. However, one notable disadvantage to the use of these bacterial exotoxins is the requirement that the toxin be internalized by the targeted cell to exert its

Abbreviations: EGF, epidermal growth factor; IL-2, interleukin 2; MBP, maltose binding protein

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toxic effects. Therefore, both the rate and route of internalization can directly impact the efficacy of the immunotoxin [16].

Here, we report the design and evaluation of a novel peptide-based immunotoxin with specificity toward ErbB2. ErbB2 is a receptor tyrosine-protein kinase that is amplified in approximately 30% of breast cancers [18] and is of strong interest in the development of targeted therapies [19]. Unlike the aforementioned immunotoxins, the toxin moiety of our peptide immunotoxin is based on a small, peptide cytolysin that can act at the plasma membrane to disrupt membrane integrity and induce cell death. Such a mechanism thereby elicits higher chances of evading the chemotherapy resistance commonly found in ErbB2-positive cancers [20,21]. Additionally, we describe a rapid, PCR-based gene synthesis strategy to engineer the NL1.1-PSA gene for *Escherichia coli*-based recombinant protein expression, suggesting that diverse *E. coli*-based genetic libraries of active immunotoxin candidates can be easily produced for activity screenings. Our findings highlight the feasibility of using small, peptide-based immunotoxins for potential targeted cancer therapies.

2. Materials and methods

2.1. Peptides used for study

All peptide candidates used for this study were synthesized by Genscript peptide services (Genscript USA, New Jersey). All peptides were obtained with >70% purity, as verified by Genscript. All peptides were diluted in DMSO in appropriate concentrations for testing prior to use and verified for solubility as necessary. Resuspended peptides were stored in −80 °C until use.

2.2. Cell culture

MCF-10A cell lines were cultured as described previously [22]. In brief, cells were grown in DMEM/F12 (Invitrogen) with 5% horse serum (Invitrogen) and supplemented with insulin, hydrocortisone, cholera toxin, epidermal growth factor (EGF), and 1% penicillin/streptomycin (Invitrogen). HACAT cells were grown in DMEM (Invitrogen) supplemented with 10% bovine serum albumin.

2.3. Retroviral transduction

The pBabe-Puro-based retroviral vector encoding wild-type ErbB2 was used to generate stable cell lines. Retroviruses were produced by cotransfection of HEK293T cells with pCLAmpho and target DNA using lipofectamine (Invitrogen). Virus was harvested and filtered at 24 and 48 h post-transfection and retroviral infections were carried out in the presence of 8 μg/mL of polybrene (Sigma–Aldrich). Following transduction, cells were selected with 2 μg/mL puromycin for two weeks. Overexpression efficiency was measured by immunoblotting.

2.4. Immunoblotting

Cells were lysed in a buffer containing 1% NP-40, 1 μg/mL aprotinin, 5 μg/mL leupeptin, and 20 μg/mL phenylmethylsulfonyl (PMSF) and cleared by centrifugation. Lysates were normalized by BCA assay (Pierce Biotechnology). The following antibodies were used for immunoblotting: β-Actin (Sigma–Aldrich), c-erbB-2 (Dako).

2.5. Cell death assay

MCF-10A cells were plated in 48-well plates and grown to approximately 50% confluence. Cells were then treated with immunotoxins at the indicated doses for 24 h and then assayed for cell death by staining with Ethidium Homodimer-1 (EthH-1, Life Technologies). Cell media was collected into eppendorf tubes and set aside. Attached cells were washed with PBS, which was then transferred to the appropriate eppendorf tube. Attached cells were then incubated in a solution of 4 μM EthH-1 in PBS in the dark at room temperature for 40 min. After 40 min, fluorescence was read with a bottom read on a SpectraMaxM5 plate reader with the following settings: excitation at 528 nm, emission at 617 nm, and a cutoff filter of 590 nm. After reading, saponin was added to each well to a final concentration of 0.1%/well to make all cells penetrable for EthH-1. The plate was then incubated in the dark, shaking gently, at room temperature for 30 min before doing a final fluorescence read as described above. Meanwhile, the collected media and PBS wash were spun down and pelleted debris and/or cells were washed once with PBS and centrifuged. The pellet was then resuspended in a solution of 4 μM EthH-1, 0.1% saponin, and PBS and transferred to a clear-bottom 96-well plate for each condition. The plate was incubated in the dark at room temperature for 15 min before being read with the fluorescence settings as described above. A percentage of total dead cells (including both attached and detached dead cells) was generated for each condition and normalized by cell type to DMSO control. *P* values were determined using a 2-tailed *t* test.

2.6. In vitro genetic construction of NL1.1-PSA immunotoxin gene via primer-based PCR

The synthesized NL1.1-PSA peptide with amino acid sequence *MYWGDSHWLQYWYEGFFALIPKIISSPLFKTLLSAVGSALSSSGGQE* was reverse translated using online software [23] and *E. coli* optimized codon tables. The gene sequence of NL1.1-PSA was determined to be 5′-ATGTATTGGGGCGATAGCCATTGGCTGCAGTATTGGTATGAAG GCTTTTTTGCGCTGATTCCGAAAATTATTAGCAGCCCGCTGTTTAAA-ACCCTGCTGAGCGCGGTGGGCAGCGCGCTGAGCAGCAGCGGCGGC-CAGGAA-3′. Ten overlapping oligonucleotide primers, 20mers (±4), were designed using Gene2Oligo software [24,25] with an average T_m of 71.75 °C. Sequences of primers used to build the NL1.1-PSA gene were:

*R*0:TCGCCCCAATACATAGGCC
*F*0:GGCCTATGTATTGGGGCGATAGCCATTGGCTGCAGT
*R*19:GCAAAAAAGCCTTCATACCAATACTGCAGCCAATGGCTA
*F*36:ATTGGTATGAAGGCTTTTTTGCGCTGATTCCGAAAATTATTA GCAG
*R*58:CAGGGTTTTAAACAGCGGGCTGCTAATAATTTTCGGAATCA GC
*F*82:CCCGCTGTTTAAAACCCTGCTGAGCGCGGTGGGCA
*R*101:CTGCTGCTCAGCGCGCTGCCCACCGCGCTCAG
*F*117:GCGCGCTGAGCAGCAGCGGCGGCCAGGAAATG
*R*133:GCTGTCCATACATACATCAGGCATTTCCTGGCCGCCG
*R*149:CCTGATGTATGTATGGACAGC

PCR using these primers was conducted initially to synthesize overlapping products including a small portion of the full-length PCR product (Settings: 95 °C for 2 min, then 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min. The average T_m of the primers was 71.75 °C). A portion of this reaction was used as a template for a second round of PCR using a set of primers (N-PSA-FWD2:5′-TACAGGGGATCCGAACTCATGTATTGGGGCGATAGCCATTGG-3′; N-PSA-REV2:5′-GACTTACTCGAGACTAGCTTATTATTCCTGGCCGCCGCT GCT-3′) designed in order to amplify the finished gene product of the desired length (183 bp). Additionally, restriction enzyme sites were incorporated into these sets of primers (FWD/BamH1 and REV/Xho1) for downstream cloning purposes, with two stop codons at the end of the sequence. Second PCR was conducted with the following settings: 30 cycles of 95 °C for 30 s, 57 °C for 30 s, and