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.

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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

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:

> R0:TCGCCCCAATACATAGGCC
> F0:GGCCTATGTATTGGGGCGATAGCCATTGGCTGCAGT
> R19:GCAAAAAAGCCTTCATACCAATACTGCAGCCAATGGCTA
> F36:ATTGGTATGAAGGCTTTTTTGCGCTGATTCCGAAAATTATTA
> GCAG
> R58:CAGGGTTTTAAACAGCGGGCTGCTAATAATTTTCGGAATCA
> GC
> F82:CCCGCTGTTTAAAACCCTGCTGAGCGCGGTGGGCA
> R101:CTGCTGCTCAGCGCGCTGCCCACCGCGCTCAG
> F117:GCGCGCTGAGCAGCAGCGGCGGCCAGGAAATG
> R133:GCTGTCCATACATACATCAGGCATTTCCTGGCCGCCG
> R149: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

A

N — [ErbB2 Ligand] — [Toxin Moiety] — C

Ligand Moieties: Toxin Moieties:

- NL1.1   - SP

- EC1   - PSA

                 - S32

B

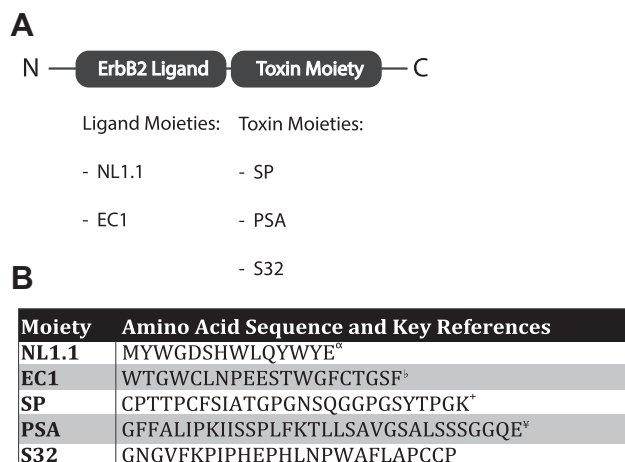| Moiety	Amino Acid Sequence and Key References
NL1.1	MYWGDSHWLQYWYE[α]
EC1	WTGWCLNPEESTWGFCTGSF[♭]
SP	CPTTPCFSIATGPGNSQGGPGSYTPGK[+]
PSA	GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE[¥]
S32	GNGVFKPIPHEPHLNPWAFLAPCCP

Fig. 1. Design of ErbB2-targeting immunotoxins. Schematic of the immunotoxin design. Any combination of one ligand moiety and one toxin moiety from the lists can be inserted into the diagram to demonstrate how the pairs can be conjugated together. Of note, the location on the amino- and carboxy-terminal ends may be exchanged to create two different immunotoxins from the same two moieties (A). Table listing the amino acid sequences of the moieties denoted in panel A (B). Please see the references associated with each of the following characters: α [28], ♭[29], + [26], ¥ [31].

72 °C for 1 min. The final PCR product was verified by Agarose gel electrophoresis and resulted in a robust band consistent with the synthesized gene (183 bp).

2.7. Cloning of NL1.1-PSA immunotoxin gene into the TOPO and pET28b vectors

The synthesized NL1.1-PSA PCR gene was cloned using primer-integrated restriction sites (BamH1, Xho1) into a modified pET-28b plasmid containing an N-terminal maltose binding protein (MBP) fusion domain as described previously [26]. Successful plasmids containing an N-terminal MBP fusion protein to the NL1.1-PSA gene were verified by sequencing. These plasmids were subsequently transformed into BL21 *E. coli* cells and used for protein induction and cell-cytotoxicity studies.

2.8. E. coli-based production of NL1.1-PSA and cell cytotoxicity evaluation

To determine cytotoxicity of *E. coli*-based production of NL1.1-PSA on host cells, HACAT and MCF-10A-derived cell lines were plated to 50–70% confluency on 12-well tissue culture plates. Cells were washed three times to remove penicillin/streptomycin-supplemented media and incubated with standard antibiotic-free media. BL21 *E. coli* containing the pET28b-MBP-NL1.1-PSA fusion gene plasmid was added from an LB diluted colony (O.D 600 ∼ 0.05) at a 1:100 dilution directly into the antibiotic-free media. IPTG was added at various concentrations (0.4 mM, 0.8 mM, 1.2 mM, 1.6 mM) directly to the wells immediately following addition of bacteria, and the plates were incubated for 4 h at in a 37 °C incubator with 5% CO_2. Following the incubation period, all wells were washed three times in cold PBS and cells were immediately counted and imaged using phase microscopy (Zeiss axiovert).

3. Results

3.1. Developing chemically synthesized ErbB2-targeted immunotoxins

Although ErbB2 has no known ligand [27], previous studies have identified small peptide fragments with a strong affinity for the ErbB2 receptor [28,29]. In our immunotoxin design strategy we therefore conjugated these ErbB2 receptor targeting ligands to several peptide sequence candidates that were previously demonstrated to have intrinsic ability to lyse cellular membranes. In particular, the three peptide toxins tested were composed of: 1. SP – a proline-substituted derivative of the Streptolysin S hemolysin from Group A *Streptococcus* that was shown to have intrinsic cytolytic activity as a peptide [30]; 2. PSA, an 18-residue peptide adapted from the N-terminus of the Pardaxin hemolysin [31]; 3. S32, a synthetically designed toxin from our bacteriocin screening library that was found to exert hemolytic activity ([26]; Lee, SW, unpublished data). Based on this template, we designed a series of immunotoxin-based synthetic peptides with two components: an ErbB2 targeting moiety and a toxin moiety. The toxin and targeting moieties were paired in various combinations, some with the targeting moiety at the amino-terminus and toxin moiety on the carboxy-terminus (Fig. 1A), others with the two moieties inverted on terminal ends. These immunotoxin peptide candidates were then synthesized as lyophilized peptides from Genscript at a >70% purity, and dissolved in DMSO prior to testing.

3.2. Identification of a toxic and specific chemically synthesized ErbB2-targeted immunotoxin

Although the targeting moieties used in the development of the immunotoxins had previously been shown to have specificity toward the ErbB2 receptor [28,29], it was not determined whether these targeting moieties would still retain specific binding interactions with their cognate receptors if they were conjugated to an additional peptide sequence. Furthermore, the cytolytic peptide domains that we incorporated into our immunotoxin strategy needed to remain active even after the incorporation of a targeting ligand. Therefore it was critical that all of the immunotoxin candidates that we produced would be evaluated both for their inherent cytotoxicity as well as targeting specificity. In order to do so, a pair of isogenic tissue culture cell lines was established. MCF-10A cells are a non-transformed mammary epithelial cell line that normally expresses very low levels of ErbB2 (Fig. 2A, MCF-10A-EV). Using retroviral transduction we engineered cells to overexpress ErbB2 (MCF-10A-ErbB2) as compared to the control (MCF-10A-EV; Fig. 2A). Using this set of isogenic cell lines, we assessed the toxicity and specificity of six synthetic immunotoxins by treating the cells with the immunotoxin and assaying for total cell death following a 24-h incubation. Three immunotoxins exhibited dose-dependent cell toxicity that was not specific to ErbB2-positive cells (Fig. 2B) and two immunotoxins exhibited very low, nonspecific toxicity (Fig. 2C). However, one immunotoxin, NL1.1-PSA, exhibited both toxicity and high specificity toward ErbB2-positive cells in a dose-dependent fashion (Fig. 2D). Of all immunotoxins screened, NL1.1-PSA was the only immunotoxin to achieve targeted action toward cells overexpressing the ErbB2 receptor.

3.3. Genetically engineered NL1.1-PSA immunotoxin gene and E. coli-based peptide expression

Numerous factors including inconsistent or loss of purity between batches, poor solubility, the need for harsh solvents, low production rate, and high cost of production, among others, can challenge synthetic production of small peptides. Given these considerations, we designed a primer-based, two step PCR method to synthetically produce the gene encoding the NL1.1-PSA candidate for cloning into an *E. coli*-based protein expression vector. A codon-optimized sequence corresponding to the NL1.1-PSA gene and overlapping primers to construct the gene sequence were designed using the Gene2Oligo software. A first round of PCR using
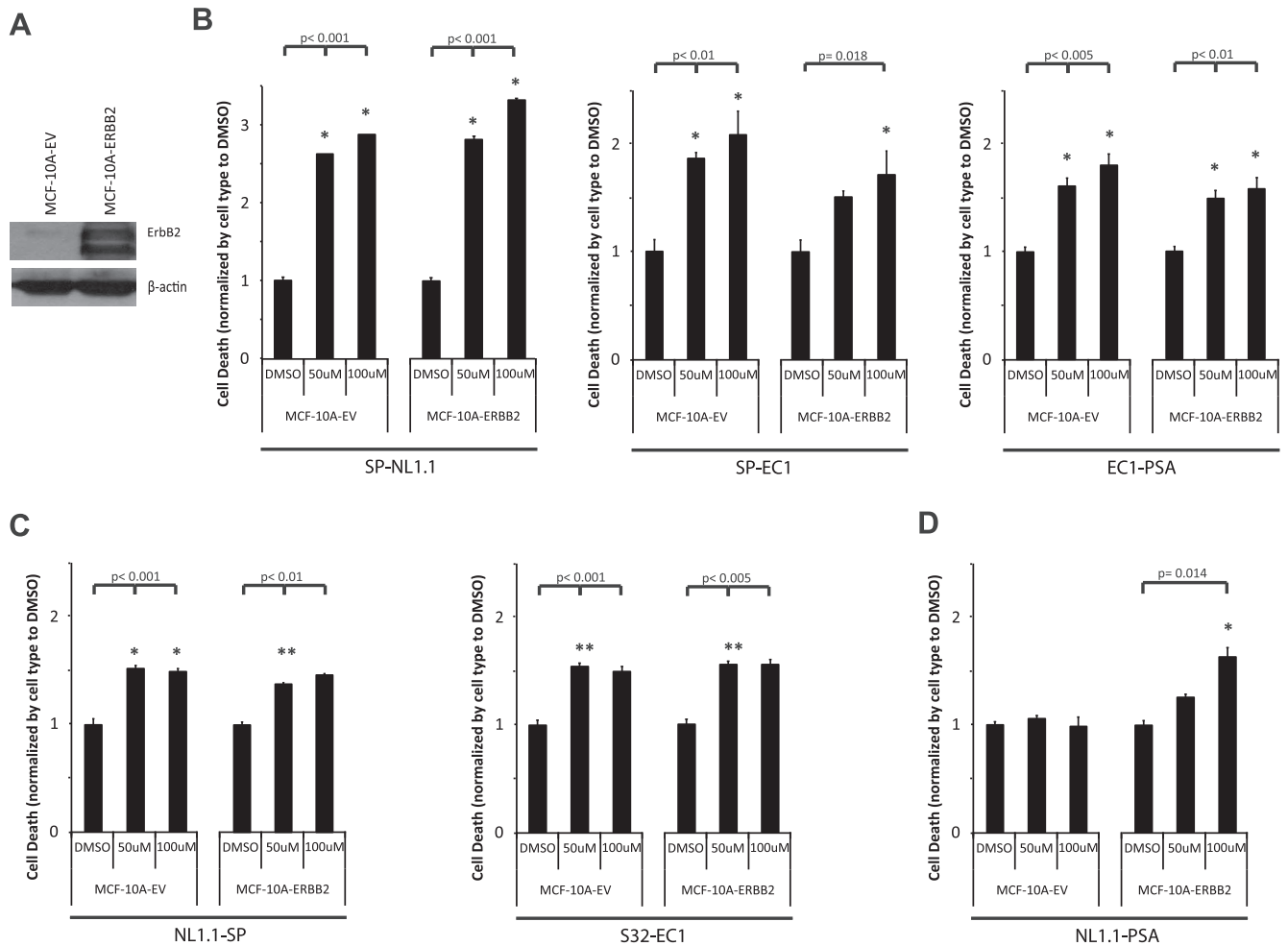
Fig. 2. The synthetic NL1.1-PSA immunotoxin is both toxic and specific to ErbB2-positive cells. Parental MCF-10A cells were engineered to overexpress ErbB2 (MCF-10A-ErbB2). MCF-10A cells transduced with virus containing an empty vector were used as a control (MCF-10A-EV). Confirmation of overexpression was done by immunoblotting. β-Actin was used as a loading control (A). MCF-10A-EV and MCF-10A-ErbB2 cells were grown to ~50% confluence and treated with the indicated immunotoxin. Cells incubated with the immunotoxin at the indicated doses for 24 h were subsequently assayed for cell death by fluorescence reading following Ethidium Homodimer-1 staining. The percentage of dead cells was normalized to the DMSO control in each cell type. Error bars represent standard deviation (B–D).

the full set of overlapping primers resulted in a small population of full-length gene sequences, which were then subjected to a second round of PCR using two additional primers designed to amplify only the full-length product (Fig. 3A). Successful clones were verified by sequencing and subsequently cloned into a pET28b-MBP fusion vector for protein expression using an IPTG-inducible BL21 *E. coli* strain (Invitrogen). Induction tests determined that a band corresponding to the correct size of MBP-NL1.1-PSA could be observed in bacterial cell lysates as well as in culture supernatants (Alber, D., Thomas, C.T., unpublished). Since production of small peptides has been known to be toxic to *E. coli*, we deduced that mature toxin might be produced and significantly accumulated in the supernatant of the culture following peptide induction. In order to test whether *E. coli* induction of MBP-NL1.1-PSA produces a secreted product that is cytotoxic, we first evaluated general cellular toxicity using HaCaT cells, a human keratinocyte cell line that expresses moderate levels of ErbB2 [32,33]. BL21 *E. coli* transformed with the MBP-NL1.1-PSA expression plasmid were added directly to HaCaT cells. As observed in Fig. 3B, NL1.1-PSA expression in BL21 *E. coli* using increasing amounts of IPTG induction (0.4 mM, 0.8 mM, 1.6 mM) resulted in increased cellular cytotoxicity, as observed by total HaCaT cells adhered to the plate. BL21

E. coli producing the MBP only control (Fig. 3B, upper panel) did not show significant cellular cytotoxicity, indicating that cell death is specific to the MBP-NL1.1-PSA toxin. To further assess the efficacy of the *E. coli*-based NL1.1-PSA product compared to the effects that we observed with the synthesized peptide (Fig. 2D), we repeated the experiment using the isogenic MCF-10A cell lines. MCF-10A-EV and MCF-10A-ErbB2 cells were plated and exposed to BL21 *E. coli* expressing the MBP-NL1.1-PSA toxin with IPTG induction (1.6 mM). Incubation of the immunotoxin-producing *E. coli* strain on these cells resulted in similarly cytotoxic phenotypes, with decreases in MCF-10A-ErbB2 cell number (Fig. 3C). However, there was a marked difference in the cytotoxic effects between the ErbB2-positive and negative cell lines, with significantly increased cytotoxicity observed in the ErbB2-positive cells (Fig. 3C). Although it is difficult to quantify the actual amount of immunotoxin produced by our *E. coli* strains during our experiment, our initial induction tests of NL1.1-PSA production showed estimated concentrations of 0.1–0.5 mg/mL total protein from a 5 mL *E. coli* culture. Taken together, these data establish both toxicity and specificity of the naturally produced NL1.1-PSA. Furthermore, these data demonstrate that immunotoxin candidates can be evaluated using an *E. coli*-based protein expression system.
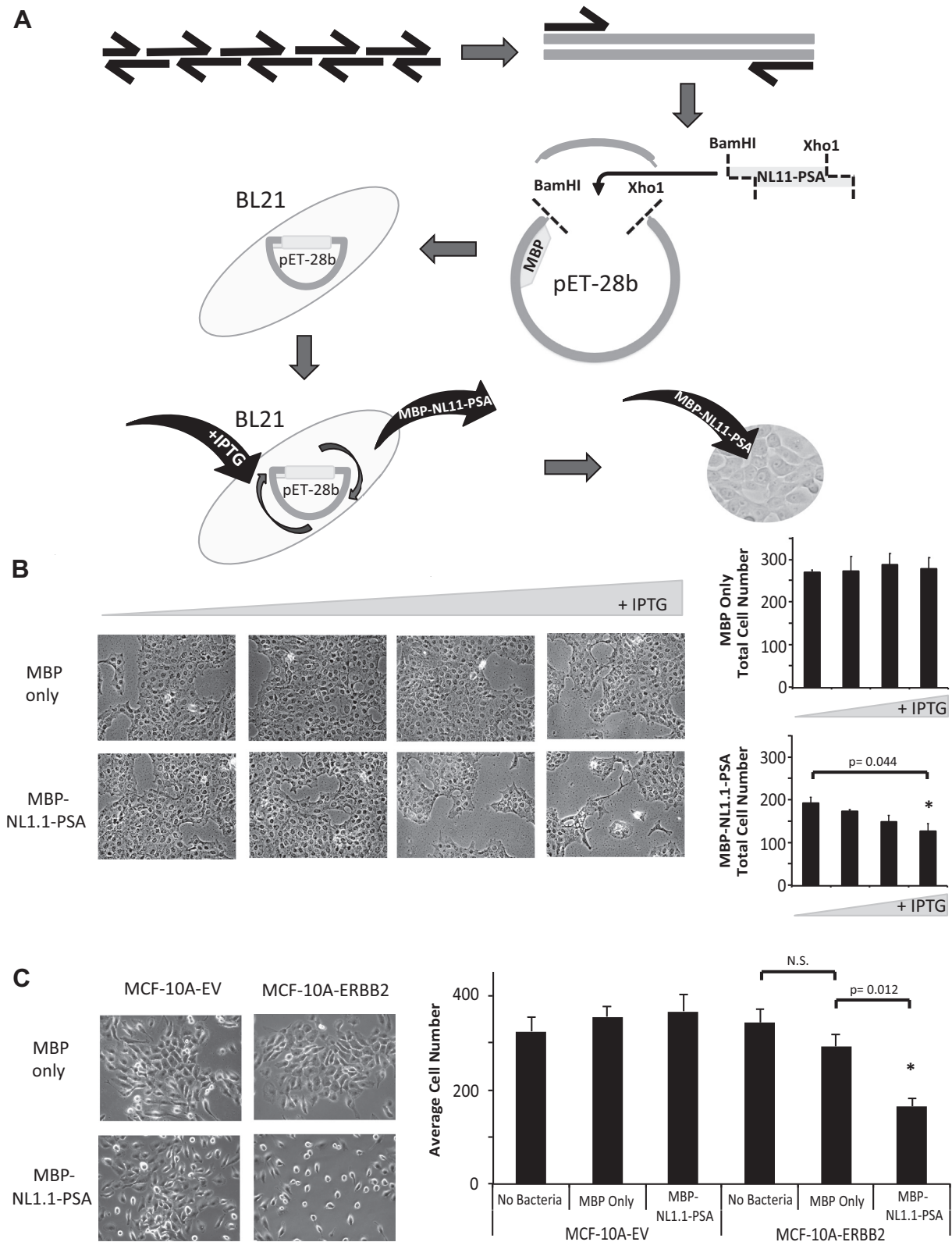
Fig. 3. *E. coli* production of NL1.1-PSA is both toxic and specific to ErbB2-positive cells. Schematic of how overlapping primers were used to build the plasmids containing the MBP-bound NL1.1-PSA sequence via PCR (A). HaCaT cells were grown to ~50% confluence and subsequently co-cultured with *E. coli* BL21 cells containing the MBP-NL1.1-PSA plasmid or an MBP-only control plasmid for 4 h. Increasing amounts of IPTG (from left to right: no IPTG, 0.4 mM, 0.8 mM, 1.6 mM) induced continuous immunotoxin protein production by *E. coli* BL21 cells. Representative images are given following IPTG induction with concentrations as follows: 0 mM (no IPTG), 0.4 mM, 0.8 mM, and 1.6 mM. Total cell number of HaCaT cells present following BL21 induction is given in graph. Error bars represent SEM (B). MCF-10A-EV and MCF-10A-ErbB2 cells were grown to ~50% confluence and subsequently co-cultured with BL21 cells containing the MBP-NL1.1-PSA plasmid or an MBP-only control plasmid for 4 h. Representative images are given following IPTG induction at a final concentration of 1.6 mM. Total cell number of MCF-10A-EV and MCF-10A-ErbB2 cells present following BL21 induction is given. Error bars represent SEM (C).

4. Discussion

Resistance to chemotherapy is a prominent barrier in treating cancer patients [1] and cancers characterized by the overexpression of ErbB2 are no exception [20,21]. As a noted receptor found enriched in cancer cells [18], ErbB2 has been an attractive target for chemotherapies. Various groups have identified unique sequences that have a strong affinity for the receptor [28,29] and utilizing modified variants of these sequences conjugated to a cytolytic peptide, we have successfully achieved specific cell killing of ErbB2-positive cells (Figs. 2D and 3C). Given that the NL1.1-PSA toxin candidate likely exerts its effects at the plasma membrane, we speculate that mechanisms of chemoresistance that involve alterations in intracellular signal transduction, up-regulation of multi-drug resistance genes, or defective vesicular trafficking would not significantly alter the efficacy of this toxin. This is in contrast to previously developed immunotoxins that require internalization for activity [8,16]. Furthermore, penetrance into solid tumors is a prominent obstacle in treatments with immunotoxins [8], likely due in part to the requirement for internalization. We have designed our cytolysin-derived immunotoxin strategy such that the capacity of NL1.1-PSA to act at the cell membrane of ErbB2-positive cell populations may serve to increase overall clearance of cancer cells from solid tumor surfaces.

Notably, a previous attempt at utilizing an ErbB2-targeting immunotoxin resulted in hepatotoxicity during clinical trials [34]. During this study, through the use of a monoclonal ErbB2 antibody, it was concluded that expression of ErbB2 in normal hepatocytes was responsible for the toxicity observed. Recent studies, however, have utilized the FDA-approved ErbB2 detection kit, HercepTest [35], to examine ErbB2 expression in patient samples and concluded that ErbB2 is minimally expressed in non-neoplastic tissues [36]. Therefore, the hepatotoxicity previously observed may be due to general off-target effects rather than the high expression of ErbB2 in hepatocytes. These data, in combination with the specificity exhibited by our immunotoxin (Figs. 2D and 3C), provide confidence that our toxin would display distinct sensitivities between ErbB2-overexpressing breast cancer cells and normal cells expressing minimal endogenous ErbB2, thereby preventing off-target toxicity.

Finally, the genetic engineering strategy presented here for the production of our peptide-based immunotoxins (Fig. 3A) allows for the opportunity to rapidly diversify toxin and ligand sequences that may have improved activity over the originally tested NL1.1-PSA toxin. Changes to the ligand binding motif using this strategy would also enable the immunotoxin to target to numerous varieties of solid tumor tissues, given a signature of overexpression markers, while still harboring the capacity to lyse the cells upon contact with the PSA toxin moiety. Hence the immunotoxin presented here is not necessarily limited to ErbB2-positive cells but could be engineered to treat a vast expanse of cancers and/or other diseases.

Importantly, we demonstrate that the peptide-based immunotoxin genetic engineering method that we describe herein could be coupled with *E. coli* induction and direct cytotoxicity evaluation using a simple tissue- culture based screen. We propose that such a method could be improved to establish a high-throughput screen for rapidly identifying new immunotoxin candidates that would have advantages in cost and time over screens using individually synthesized peptides.

Overall, we acknowledge that further studies are required to explore the clinical application of the NL1.1-PSA immunotoxin presented here, including the use of 3-dimensional *in vitro* assays to more closely simulate tumor environments *in situ* as well as efficacy using *in vivo* animal models. However, our strategy to design a small, peptide-based immunotoxin that exerts its toxic effects at the cell surface of cancer cells holds immense promise in overcoming the resistance to chemotherapy as either a first or second line treatment option. These data open the door to future research aimed at eliminating solid tumors in a way that minimizes resistance to treatment and enhances overall patient survival.

5. Conclusions

ErbB2 is a receptor tyrosine-kinase that is overexpressed in the tumor cells of approximately 30% of breast cancer patients, and cancer cells with ErbB2 overexpression frequently acquire resistance to traditional chemotherapies. This substantiates the need for the development of additional, specific therapeutics targeting ErbB2-positive cells. Here we describe the development and testing of a peptide-based immunotoxin, NL1.1-PSA, that exhibits ErbB2-specific cytotoxicity. The utilization of the immunotoxin described here could overcome the likelihood of chemoresistance and thereby more effectively eliminate ErbB2-positive breast cancer cells. We also present an *E. coli*-based bioengineering approach for the production and testing of peptide immunotoxin candidates. This would enable future research to expand on our findings with ErbB2-positive cells to engineer immunotoxins with targeting specificity toward other receptors and markers of interest.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

SWL and ZTS conceived of the original design and testing of the immunotoxin candidates. KJW directed the experimental design, testing, and evaluation of the peptide immunotoxins. LS, LD conducted cytotoxicity studies with KJW. DA and CLT conducted the *E. coli* based engineering immunotoxin studies. KJW and SWL wrote and edited the manuscript and designed the figures for publication. All authors contributed to the final editing of the manuscript prior to submission.

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