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Award Number: DAMD17-96-1-6208

TITLE: Engineering Diptheria Toxin Towards the Development of Therapeutics Against Breast Cancer

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REPORT DATE: September 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3 REPORT TYPE AND DATES COVERED
	September 1999	Final (1 Sep 96 - 31 Aug 99)

4. TITLE AND SUBTITLE
Engineering Diptheria Toxin Towards the Development of Therapeutics Against Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6208

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

We have completed the crystal structure determination of diphtheria toxin bound to its normal receptor (Louie et al., Molecular Cell, 1, 67-78, 1997).

The crystal structure of diphtheria toxin (DT) complexed 1:1 with a extracellular fragment of its cell-surface receptor. The toxin receptor is a membrane-bound precursor of a heparin-binding EGF-like growth factor (HB-EGF). The structure reveals that a ß-sheet in the receptor-binding domain of DT packs against a ß-sheet of the EGF-like fold.

Using a three-dimensional picture as a guide, we have identified key atoms dictating the fit between toxin and receptor and remodify the toxin to target to heregulin, whose overexpression is correlated with the onset of metastatic breast cancer.

Future studies will include testing the function of this recombinant toxin in cell culture systems of breast cancer cell lines.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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S. Moe 9/30/99

PI - Signature Date

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INTRODUCTION

The goal of this project is to design a cytotoxic protein using diphtheria toxin as a template that will recognize and bind to a cell that overproduces heregulin (HRG) precursor on the cell surface. HRG is an activating growth factor ligand for HER-4, whose overexpression is correlated with breast cancer cells. Given the recent success with the humanized monoclonal antibody (herceptin) against erb-B receptors as therapeutics against breast cancer, it is reasonable to hypothesize that the downregulation of the production of this ligand may lead to the inhibition of the growth of breast cancer cells. HBEGF is structurally homologous to HRG, thus providing a point of departure for molecular design of diphtheria toxin in order to divert to a new target receptor, HRG.

A single molecule of diphtheria toxin of 535 amino-acid residues is sufficient to kill a cell. The killing action of DT involves three distinct steps. First, it binds to a receptor on the surface of sensitive cells and subsequent receptor-mediated endocytosis. Second, it translocates the catalytic domain of the toxin across the endosomal membrane and into the cytoplasm of the cell, a process induced by the acidic environment inside the endosome. Third, the catalytic transfer of an ADP-ribosyl group from NAD^+ to elongation factor-2 by the toxin domain prevents protein synthesis in the cell and leads to cell death. In the earlier part of this project, we have completed the crystal structure of diphtheria toxin (DT) complexed with an extracellular fragment of its receptor protein, a membrane-bound precursor of human heparin-binding EGF (hHBEGF). Since then, a good part of our effort was focussed to make an engineered DT that will target to a different receptor. A modified HBEGF was used as a control, to which combinatorial mixture of DT was first screened. The modification of HBEGF was based on the fact that mouse HBEGF (mHBEGF) are not receptors for DT, whereas hHBEGF is. Between hHBEGF and mHBEGF at the DT-facing interface, there are about 10 amino acids that are different to act as the first set of candidate to encode such a different binding affinity. Our main effort has been to establish the recombination, screening and assay methods in order to utilize those sites to recreate a new binding interface. We have not been able to provide a convincing example to demonstrate it, yet have learned about a number of potential problems along the way.

BODY OF RESULTS

Structural Basis of the DT-HBEGF Interface

The comprehensive structural analysis as described in Louie et al. (1997) illustrates that the structural scaffold of HBEGF is structurally homologous to that of HRG. The structure of the DT/HBEGF complex is, therefore, an ideal candidate to model the binding interface of HRG with the structure of HBEGF as template. Eleven interface positions have been selected for site-specfic mutagenesis as prime candidates for binding specificity-determining residues. These interactions are predominantly nonpolar at the center and is surrounded by polar interaction. Non-polar sidechains involve Phe_{389}, Ala_{430}, Leu_{433}, Ile_{464}, Val_{468}, Phe_{470}, Gly_{510}, Leu_{512}, Val_{523}, and Phe_{530}

of DT or Val_{124}, Leu_{127}, Ala_{129}, Pro_{130}, Ser_{131}, Ile_{133}, and Pro_{136}, the Gly_{137} and the Cys_{134}-Cys_{143} disulfide bond of HBEGF. In the periphery of the nonpolar core of the interface are a number of direct hydrogen-bond interactions (17 in total) involving a number of main-chain polar groups. All these factors together with the direct and water-mediated interactions undoubtedly contribute to the specificity of DT for hHBEGF with the relatively tight binding affinity ($K_d \sim 10^{-8}$ M).

To develop the redesign protocols, we chose to create an easy target first: hHBEGF was modified by a single amino acid change at 141 from Glu to His. This is the amino acid which was found to be most extensively involved in H-bonding, and was also identified as the most critical residue for DT binding from alanine-scanning experiments. In the context of the DT/hHBEGF complex structure, Glu141 is hydrogen-bonded with two basic sidechains of DT: His391 and Lys516. Asp392 of DT is also indirectly involved by packing against His391. In mHBEGF, the amino acid at this position is His. Thus a tentative interpretation is that His occupying the same position would provide a repulsive interaction with these neighboring DT amino acids, creating an energy barrier for favorable binding. So hHBEGF(E141H) provides a fairly reasonable challenge to simulate the alteration of binding specificity because all other amino acids will be preserved as those of hHBEGF.

With the aim to restore favorable H-bonding interactions with hHBEGF(E141H) interface, we have introduced randomized codons encoding six polar amino acids (His, Gln, Asn, Lys, Asp, or Glu) at each of the 391, 392, and 516 positions of DT. The diversity is encoded in libraries of oligonucleotide sequences used for PCR, in which the codon triplet for each of these three positions is ($A_1/C_1/G_1$. A_2. C_3/G_3). The libraries were introduced into the entire DT or only the receptor-binding domain of DT (R-domain). The modified HBEGF, like wild-type, was overexpressed as a GST-fusion protein to facilitate protein folding of the EGF module and to use GST as an affinity marker.

To express the randomized DT sequences, we have explored three different systems: 1) fusion of DT to an ice-nucleating protein to display on the cell surface of E. coli, 2) expression of DT in lysogenic plaques of bacteriophage lambda, and 3) phage display system. These approaches all require a common procedure, for which we have not been able to demonstrate a reproducible result: quantitate the binding. To detect the binding, we have used blotting the colonies onto a detection medium and incubate with appropriate detection tools primarily goat-antibody for GST, a secondary anti-goat antibody (conjugated with horse radish peroxidase HRP) and chemiluminiscent reagents to stain for HRP. With the first system (ice-nucleating fusion on E. coli surface), we have not fully confirmed the level of cell surface expression as ice-nucleating fusion protein and did not follow up subsequently. All approaches are prone to diminished sensitivity and high background arising from nonspecific binding. Further optimization would ensure a better result. Nevertheless, the most promising demonstration of target-specific selection has been obtained by the third method (phage display). In this method, the DT-encoding DNA sequence is expressed as part of C-terminal capsid fusion protein and displayed on the phage particle. Phages capable of

binding the target (hHBEGF(E141H) for instance) can then be isolated by first mixing the phage library with the target protein, adsorbing to GST resin, washing to remove unbound phages, and elute the bound phages with GST, repeated several times. The selected phages are amplified by infecting into E. coli, let lysed from the bacteria, and havested for sequencing for identification of the given DT sequences in it.

Eight colonies from the batches selected against hHBEGF(E141H) or wild-type hHBEGF were sequenced and compared. With wild-type, they are (Q,D,K), (H,D,K), (Q,D,K), (N,D,K), (K,H,K), (Q,N,H), (K,H,K), and (K,K,H) for positions 391, 392, and 516, respectively. This set contains invariably a basic sidechain at 516, perhaps to complement Glu141 in the target protein. It also includes the wild-type DT sequence (#2: H,D,K). In contrast, with the hHBEGF(E141H), they are (D,Q,H), (E,Q,Q), (H,N,E), (D,E,Q), (H,D,K), (D,H,K), (H,N,E), and (Q,N,E). Ironically, one (#5) corresponds to wild-type sequence of DT. This screening was performed with R-domain constructs alone. When these gene segments were trasferred into the entire DT constructs, those sequences selected for the modified hHBEGF (#2,3, and 8) in DT did not show appreciable specific binding as compared to the wild-type HBEGF.

Important considerations with the phage-display approach are the possibility of overamplification of those with wealk binding, and poor discrimination at the step of selection, which will result in higher level of non-specific binders in the final pool. We decided that we need further verification and establish the method with possibly alternate binding pairs of proteins for future experiments.

KEY RESEARCH ACCOMPLISHMENTS

- Similarity of HBEGF to other EGF-like proteins

- Atomic Details of toxin binding to HBEGF

Similarity of HBEGF to other EGF-like proteins
We have determined and showed a common structural EGF scaffold for the HBEGF receptor from the DT-HBEGF complex. It consists of a long and short ß-hairpin stabilized by three disulfide bonds. A superposition of HBEGF with other EGF modules reveals that the conformations of the major secondary structural components and the three disulfide bonds are in good agreement. We found that the largest structural differences among the various EGFs occur within two loops that links the two strands of the major ß-hairpin. The overall average positional deviation between HBEGF and α-HRG is only 1.26 Å for 35 residues in the core.

Atomic Details of toxin binding to HBEGF
We have described at atomic details how the toxin recognizes and binds to HBEGF receptor. For instance, the most critical substitution in mouse HBEGF is Glu141His of human HBEGF, which would potentially place three basic side chains of DT (His_{141}, His_{391} and Lys_{516}) in close proximity in the complex. These observations are consistent with results from changes made at the site. First, the Lys516Ala of DT mutant shows

the largest decreases in toxicity and receptor-binding affinity. Second, chimeras of mouse HBEGF with either the human or monkey forms show that the single amino-acid substitution Glu141His is sufficient to preclude almost all DT binding. Nevertheless, binding determinants besides Glu_{141} should be also important, since mouse HBEGF with the single His141Glu mutation shows only slight binding to DT.

The strong structural similarity between HBEGF in the complex and other EGF modules both in solution and in crystals (from our work) has an important implication for future studies. The receptor-binding domain of the DT molecule has been adapted to bind HBEGF such that little distortion occurs relative to the uncomplexed form of the receptor. Therefore, other EGF modules can make feasible recognition targets for engineered DTs with redesigned binding specificities. With HRG as an ultimate target, those positions surrounding the key determinants of the HRG receptor can be evaluated by measuring the binding affinity of various DT mutants individually.

REPORTABLE OUTCOMES

To establish the protocols for selection from randomized sequences at these sites and to demonstrate the concept of principle, we chose mouse HB-EGF as a first target to switch the binding affinity. One primary site was chosen to create mouse-like HBEGF. Three key positions of DT have been selected Asp_{392}, Lys_{516} and His_{391} of DT from the DT/receptor binding interface that define the binding specificity against this particular amino acid (Glu141 to His of mHBEGF). These selected sites have been randomized by PCR-based mutagenesis systematically to polar sidechains and mutants screened for their binding affinity and specificity have been tested for the specific binding. Disappointingly, these selection protocols did not pick up a sequence with high specificity. It did not discriminate against wild-type sequence. We conclude that binding assay systems need further development. Thus we could not reach the ultimate task of testing against HRG. As the methods become established and available, we will be able to expand our targets, ultimately to HRG as the ultimate target.

CONCLUSIONS

As a first step towards the goals, most efforts have been made to establish the protocols of randomizing codons at multiple locations within DT and to screen for those that have altered binding specificity without convincing results. From our experience, it has been most effective to use virus-based vectors (phage display system). The ambiguity in the binding data analysis may come from the selection protocol. It can be overcome by experimenting with carefully selected positive and negative controls to assess the validity of the assay methods individually.

Alternatively, it might be feasible to screen for colonies using fluorescence-activated cell sorters after cells are labeled by fluorescence-tagged receptor fragment of HRG or colony screening with fluorescence-tagged receptor protein to speed up the screening. Such assay system would require the uniform expression of the modified DT protein on

the cell surface. Fusion with ice-nucleating enzyme on E. coli surface is an excellent candidate for such display. The mutant identified with the highest affinity from this screening can then be tested subsequently for its efficacy in vivo cell culture individually.

REFRERENCES

Louie, G., Yang,W., Bowman, M., Choe, S. (1997) Crystal structure of diphtheria toxin in complex with its receptor. *Molecular Cell*, 1, 67-78.

APENDICES

A reprint is attached.

FINAL REPORTS

The publication during the funding period is listed under references as above. Two personnel have been supported for this work: Dr. Gordon Louie, and Mr. Walter Yang. Both are coauthors of the paper, Louie et al. (1997). Dr. Louie has continued to pursue the goal of protein engineering the toxin molecule in order to modulate its target specificity from human HBEGF to heregulin. The ultimate test will be whether such engineered toxin will be of therapeutic value in the treatment of breast cancer. However, as of the termination date of the proposed work, we have not convincingly demonstrated the feasibility of the recombination, screening, and assay methods. Further pursuit to target towards HRG, therefore, should be delayed.