California State University Northridge

Chimeric Anti-Cancer Proteins Engineered for Delivery by Tumor-Targeted Salmonella

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By

David A. Quintero

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The thesis of David A. Quintero approved by:

______________________________
Dr. Michael Summers
Date

______________________________
Dr. Kerry Cooper
Date

______________________________
Dr. David Bermudes, Chair
Date
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# TABLE OF CONTENTS

[Signature Page](#) ii

Acknowledgment iii

List of Figures viii

List of Tables x

[Abstract](#) xi

[Introduction](#) 1

- History of live bacterial therapeutics 1
- Cancer prevalence and resistance mechanisms 3

*Salmonella typhimurium* str. VNP20009 4

*Pseudomonas aeruginosa* Exotoxin A (ToxA) 8

Chimeras of ToxA 10

Epidermal growth factor receptor (EGFR) 13

Expression of ToxA chimeras by tumor-targeted *Salmonella* 14

[Materials and Methods](#) 16

- Fusion Model of a TGFα:PE40 Chimera 16
- Bacterial Strains and Plasmids 17
Development of the novel bacteriological media CTY

Preparation of electro-competent bacteria using a plate method

PCR and Cloning

Overlapping PCRs

Cloning of the wild type ToxA and the pColE3 lysis protein

Gene synthesis and cloning of TGFα:ToxA chimeras

Construction of OmpA:TGFα:3GS:Dlb:DIiIK (O-T-G-DlbK) and OmpA:TGFα:3GS:Dlb:DIiIR (O-T-G-DlbR)

Construction of OmpA:TGFα:3GS:DIiIK (O-T-G-DIIIK) and OmpA:TGFα:3GS:DIiIR (O-T-G-DIIIK)

Construction of OmpA:TGFα:3GS:PE38K (O-T-G-PE38K) and OmpA:TGFα:3GS:PE38R (O-T-G-PE38R)

Insertion of the pColE3 lysis protein sequence for co-expression

Deletion analysis of O-T-G-PE38K

Tumor cells

Cell proliferation and cytotoxicity assays

Protein detection and quantification

JC-10 mitochondrial membrane depolarization assay

Detection of bacterial membrane disruption using a phosphatase assay
Results 38

Theoretical model of a TGFα:PE40 chimera 38

Development of the Novel Bacteriological Media CTY 40

Quantification of VNP20009 produced WT ToxA and relative production of TGFα:ToxA chimeras using an anti-ToxA antibody 42

Relative toxicities of the TGFα:ToxA chimeras 44

Quantification of the TGFα:ToxA Chimeras 46

IC$_{50}$ comparisons of the KDEL substitution and co-expression of the pColE3 lysis protein relative to WT ToxA and O-T-G-PE38R 48

Deletion Analysis of O-T-G-PE38K 50

O-T-G-PE38K is selective for an EGFR over-expressing cell line 53

Evaluation of the KDEL substitution and co-expression of pColE3 lysis in the O-T-G-DIbR and O-T-G-DIIIR constructs 57

Detection of bacterial outer-membrane disruption using a phosphatase assay 59

Discussion 61

Summary of results 61

Future directions 71
Appendix: Computer program for fusion of TGFα:PE40 in Python language
LIST OF FIGURES

Figure 1: Theoretical model of a TGFα:PE40 chimera 39

Figure 2: Assessment of bacterial media components on mammalian cell survival 41

Figure 3: Comparison of the wild type ToxA and ToxA chimeras 43

Figure 4: Dose-response curves of wild type ToxA and ToxA chimeras 45

Figure 5: Quantification of the chimeric proteins present in culture supernatants and their relative cytotoxicities 47

Figure 6: Relative culture supernatant toxicities of wild type ToxA and chimeric variants 49

Figure 7: Deletion analysis of the O-T-G-PE38K N-terminal components and comparisons with the E553D substitution and the co-expression of the ColE3 lysis protein 52

Figure 8: Relative expression levels of epidermal growth factor receptor (EGFR) in different tumor cell lines and the relative sensitivities of those cell lines to culture supernatants of VNP20009 expressing either WT ToxA or O-T-G-PE38K 55

Figure 9: JC-10 Mitochondrial depolarization assay 56
Figure 10: Effect of the addition of KDEL and pColE3 Lysis in O-T-G-DIbR and O-T-G-DIIIR

Figure 11: Phosphatase assay on bacterial supernatants expressing WT ToxA and TGFα:ToxA chimeras
LIST OF TABLES

Table 1: Bacterial strains 33

Table 2: Cloning schematic, plasmids, primers, and restriction enzymes 34

Table 3: Sequencing primers 37
Chimeric Anti-Cancer Proteins Engineered for Delivery by Tumor-Targeted *Salmonella*

By
David A. Quintero
Master of Science in Biology

Salmonella str. VNP20009 selectively colonizes solid tumors and has been shown to reduce their growth in mouse models. Studies have demonstrated that Salmonella can induce apoptosis through direct contact with cells. However, this property limits the potential of the bacterial therapy by requiring physical association with tumor cells. In order to overcome this limitation, these bacteria can be engineered to express and secrete cytotoxins that are tumor cell specific, which may enhance tumor destruction while sparing healthy adjacent tissues. Epidermal growth factor receptor (EGFR) is overexpressed in many cancer cell lines including breast, prostate, colon, brain and others, and is a validated target in the clinical setting. In this thesis, EGFR-targeting toxins were engineered and assessed for their ability to be secreted or released into the extracellular milieu by VNP20009. VNP20009 was able to secrete or release three forms of a truncated *Pseudomonas aeruginosa* Exotoxin A (ToxA) cytotoxin that was fused to...
the EGFR-binding ligand, tumor growth factor-alpha (TGFα), which resulted in bacterial culture supernatants that were selectively cytotoxic to cells overexpressing EGFR. Additionally, the native ToxA C-terminal endoplasmic reticulum (ER) retention signal was replaced with the mammalian consensus sequence KDEL to enhance toxicity, and were co-expressed with the pColE3 lysis protein to facilitate secretion. These genetic manipulations resulted in a supernatant that is more active against EGFR positive cells than the secreted wild type ToxA, and may translate into a potent, targeted bacterial therapy.
INTRODUCTION

**History of live bacterial therapeutics**

The use of live bacteria for therapeutic purposes has gained much attention recently due in part to work on the human microbiome and the roles commensal bacteria play in human health (Hsieh and Versalovic, 2008). The results of such reports have bolstered the use of these beneficial bacteria and their metabolic byproducts in the form of probiotics and prebiotics, while some have called for the construction of genetically modified bacterial strains with enhanced therapeutic activities (Chen et al, 2014). Genetically enhanced bacteria may act as a vector for delivery of therapeutic compounds directly within diseased tissues and are currently being investigated by laboratories around the globe. These approaches include engineered bacteria for the treatment of autoimmune and inflammatory disorders, metabolic disorders, antibacterial and antiviral therapies, gene editing, and cancer therapeutics with sustained delivery (Piñero-Lambea et al., 2015), underscoring the innumerable possibilities and unlimited potential for the use of live therapeutic bacteria.

The concept of live bacteria as a potential cancer therapeutic dates back to the early 1800’s when Vautier first reported that tumor regression had occurred in patients with inoperable tumors whom had simultaneously suffered from an incident of gas gangrene, caused by the then unknown bacillus *Clostridium perfringens* (Wei et al, 2008). Later, the German physicians Busch and Fehleisen observed that an outbreak of erysipelas, induced by an infection by *Streptococcus pyogenes*, also resulted in tumor
regression (Patyar et al, 2010). Subsequently, the American physician Coley treated individuals affected by inoperable tumors with live *Streptococcus* and found that it was the fever associated with the infections that led to tumor regression; a process we now understand to be host production of tumor necrosis factor-alpha (TNFα) and the resulting immune response triggered by the presence of the bacteria.

Utilizing live bacteria posed many difficulties, including uncontrollable infections or inconsistencies in mounting an immune response (fever), therefore Coley continued his studies utilizing a mixture of bacterial extracts derived from *Streptococcus pyogenes* and *Serratia marcescans*, known as Coley’s toxin. This aided his studies through removal of the infectious agent, while maintaining the capacity to induce the fever associated with tumor regression in a controllable manner. The outcome of those studies led to complete or near complete regression of some solid tumors, namely sarcomas, and others used similar extracts to treat different tumor types such as carcinomas, lymphomas, melanomas and myelomas with some success (Cann et al., 2003). These findings led to explorations in the use of live attenuated bacteria to treat cancer and laid the foundation for modern attempts with clinical applications encompassing engineered bacterial strains across many genera including *Clostridium* (ClinicalTrials.gov Identifier NCT01118819) *Listeria* (ClinicalTrials.gov Identifier NCT01598792) and *Salmonella* (ClinicalTrials.gov Identifier NCT01099631; ClinicalTrials.gov Identifier NCT00004988).
Cancer prevalence and resistance mechanisms

Cancer remains a global health concern with approximately 1.5 million new cases projected every year and one-third of those projections leading to mortality on a yearly basis. In the United States, cancer as a cause of death is second only to heart disease and, with the advent of prescription drugs that minimize cardiovascular related incidents, these statistics are now shifting towards cancer with 21 states recently reporting that cancer is the primary cause of death (Siegal et al., 2016).

There are several factors that contribute to the difficulty of treating cancer that range from the limited bio-distribution of cancer drugs due to bio-physical constraints, to phenotypic differences between cancer types that lead to drug resistance. Tumors for example, are characterized as having large interstitial spaces, which act as reservoirs for extracellular fluid, and generally have collapsed lymphatic vessels, which are responsible for draining the interstitial compartment (Jain 1987 and Leu et al, 2000). This combination causes an increase in interstitial pressure that hinders penetration and delivery of anti-cancer compounds into the tumor (Azzi et al., 2013). The majority of cancer drugs target fast growing cells that are usually found at the periphery of a tumor and thus quiescent cells that are located centrally to the tumor and distal from the vasculature can go unscathed (Kasinskas et al, 2006).

Lack of perfusion is especially important in terms of inducing a drug resistant phenotype through inadvertent selection of tumor cells with the mechanisms in place to circumvent cell toxicity. Various tumor types have shown drug resistance through mechanisms that include the expression of efflux pumps, alteration in membrane permeability, downregulation of targeted receptors, and either increasing production or
modifying protein targets (Gottesman, 2002). These complications related to drug
distribution indicate the need of alternative drug delivery systems, preferably one which
may provide a sustained and concentrated supply of an anti-cancer compound(s) directly
within the tumor.

*Salmonella typhimurium str. VNP20009*

There have been several documented cases of *Salmonella* infections of solid
tumors ranging from colonization within liver tumors to infection of glioblastomas of the
brain (Simmers et al., 1997 and Noguerado et al., 1987). Exactly how the bacteria
accumulate in tumors remains unclear, though several independent studies suggest tumor
targeting is a multifactorial mechanism encompassing bacterial metabolism and motility,
host immunity, nutrient availability, and tumor specific chemotactic compounds (Wall et
al., 2010).

As facultative anaerobes, *Salmonella* can survive in hypoxic or anoxic
environments permitting growth within the low oxygen containing tissues of the tumors.
Solid tumors provide a barrier to immune cells and their effector proteins through limited
circulation within the tumor and may contribute to *Salmonella’s* selective accumulation
by providing a zone of protected growth (Forbes et al, 2003). Recent data suggests that
*Salmonella* produce a protective biofilm layer within necrotic tissues of the tumor
providing themselves with a defensive barrier against immune cells (Crull, et al., 2011)
Bacterial expression of aspartate, serine, and ribose/galactose receptors and the
availability of their respective ligands within the tumor seem to play a role in tumor
chemotaxis, while specific surface glycans expressed by cancer cells have also been shown to attract the bacteria (Kasinskas and Forbes, 2007 and Wang et al., 2016). Other targeting mechanisms remain to be elucidated and are being pursued for the purposes of engineering an idealized tumor-selective bacterial strain.

VNP20009, is a highly attenuated strain of *Salmonella typhimurium* that has been shown to target solid tumors and produce anti-tumor effects in mice models using mouse and human tumor cell xenografts (Luo et al, 2001). VNP20009 carries three key mutations that enhance its targeting abilities and stabilize its growth kinetics, while dramatically increasing its safety profile in terms of host toxicity (Clairmont et al, 2000). Deletion of the *purI* gene that abolishes prototrophic purine production, requires the bacteria to obtain the nutrient from an exogenous source, and was shown to increase the tumor colonization of *Salmonella*, while decreasing its virulence and off-target accumulation in healthy tissues (Pawelek et al., 1997). Targeted localization of the bacteria increased from a tumor to liver ratio of 300:1 in WT to 1000:1 in the purine auxotroph, providing evidence that preferential accumulation of the strain within the tumor may be due to the availability of free purines at the tumor site and that targeted-*Salmonella* as a bacterial cancer therapeutic may benefit from such mutations.

A major disadvantage of using *Salmonella* as a therapeutic is the presence of the endotoxin, lipid A. Lipid A is a structural component of lipopolysaccharide (LPS) that acts as an anchor for LPS via interaction of its fatty acids, myristate, laurate, and palmitate, within the outer-membrane bilayer (Karsten et al., 2009). Fully functional LPS is highly inflammatory in mammals, initiating an immense immune response through binding the CD14 or TLR-4 receptor on immune cells leading to signal transduction.
Signal transduction leads to activation of NFκB and induction of TNFα production (Raetz and Whitfield, 2002), which in turn activates production of cytokines that can cause extreme vasodilation, triggering a critical drop in blood pressure that can end in life-threatening organ failure.

Partial deletion of the msbB gene, that is responsible for decorating lipid A with a myristate group, causes non-myristoylated LPS production and was shown to decrease sepsis in mammals infected with the Salmonella strain (Low et al., 1999). This mutation leads to a significant reduction in TNFα production, requiring 10,000 fold more mutated LPS than wild type to induce a response in vitro. Salmonella msbB mutants have reduced growth rates and are highly sensitive to salts, galactose-MacConkey and the divalent cation chelator, ethylene glycol-bis(b-aminoethyl ether)-N,N,N9,N9-tetraacetic acid (EGTA) as well as acidic pH (Murray et al, 2001). Growth media lacking salt and supplemented with Ca\(^{2+}\) or Mg\(^{2+}\) has been reported to stabilize the mutant phenotype, possibly through interaction with the negatively charged phosphates of the outer membrane. Salmonella msbB mutants retained their targeting abilities, but exhibited reduced survivability within macrophages, presumably due to reduced outer-membrane function through the non-myristoylated LPS and intolerance of the highly acidic environment within the macrophage vacuole (Low et al., 1999).

Spontaneous suppressor mutants of the msbB phenotype contained a disruption in somA (function unknown) resulting in faster growth rates and resistance to salt, MacConkey, and EGTA, that occurred at frequencies as high as 9.9 \(\times\) 10\(^{-3}\) when grown in Luria broth (LB) (Murray et al., 2001). A purI msbB double mutant was genetically stabilized by introducing a spontaneous msbB suppressor mutation, known as the...

Suwwan deletion, that resulted in a recombination event between two IS200 insertion elements, causing a 108 kb deletion that included somA, and constitutes the complete construction of tumor-targeting *Salmonella typhimurium* str. VNP20009 (*purF*, *msbB*, Suwwan deletion) (Murray et al, 2004 and Broadway et al, 2014).

In a Phase I clinical trial, intravenous administration of VNP20009 was shown to be safely tolerated and demonstrated the ability of the bacteria to target solid tumors in melanoma patients, but did not show tumor regression as was seen in mice (Toso et al., 2002). In an attempt to increase anti-tumor activity, VNP20009 has been engineered to constitutively express enzymes for pro-drug conversion to active anti-tumor drugs. TAPET-CD, a derivative of VNP20009 with a chromosomal insertion of the *E. coli* cytosine deaminase (CD) gene that converts the prodrug 5-Fluorocytosine (5-FC) to the active cancer drug 5-Fluorouracil (5-FU), was shown to process the conversion to the active drug at the tumor level in a human pilot trial (Nemunaitis et al, 2003). In a similar application, the *Salmonella* purine nucleoside phosphorylase (sPNP) enzyme, that is naturally and constitutively expressed by VNP20009 without genetic modification, was utilized for the conversion of the prodrug 6-methylpurine 2’-deoxyriboside (6MePdR) into the cancer drug 6-methylpurine (6MeP) (Chen et al., 2013). Infection by VNP20009 in the presence of 6MePdR significantly reduced tumor volume in mice when compared to bacterial infection or prodrug treatment alone. Recently, bacterial derived cytotoxins such as *S. aureas* α-hemolysin (SAH) and *Pseudomonas* Exotoxin A (ToxA) have been successfully expressed and secreted by VNP20009 with high toxicity towards tumor cells and have been proposed as a method of arming the bacteria where primary localization
within a tumor can lead to relatively high concentrations of toxin within target tissues. (Swofford et al, 2014).

*Pseudomonas aeruginosa* Exotoxin A (ToxA)

Exotoxin A (ToxA) is a secreted toxin derived from the bacterium *Pseudomonas aeruginosa* that has undergone extensive study in both *Pseudomonas* and *E. coli*. The complete precursor ToxA protein sequence consists of a secretion signal (ToxA SS), a binding domain (DIa), a translocation domain (DII), a domain with an unknown function (DIIb), and an enzymatically active domain (DIII). The ToxA SS is a typical Type II secretion signal composed of 25 amino acids consisting of N-terminal positively charged amino acids followed by a stretch of hydrophobic residues and ending with a peptidase cleavage site between two alanines. Cleavage yields the mature active toxin with a molecular weight of 66 kDa (Lorey et al, 1988) (Figure 1, upper panel). The mature protein begins with the N-terminal domain DIa (amino acids +1 to 252), that binds to the low density lipoprotein receptor-related protein (LRP) receptor in mammalian cells and is required for the internalization of the wild type toxin through receptor-mediated endocytosis.

Downstream of DIa is domain DII (amino acids 253 to 364), that has been identified as the translocating domain, which facilitates escape of the toxin from the endosome into the golgi apparatus (Siegall et al, 1989). DII contains a furin recognition site RHRQR (amino acids 276-279) that lies between a cysteine bond (C^{265} - C^{287}) and is cleaved to essentially separate a 28 kDa N-terminal fragment (DIa and partial DII) from
the remaining 37 kDa C-terminal fragment containing the active toxin domain. Interestingly, the presence of the furin site has been associated with increased toxicity and is required in some mammalian cell lines (Inocencio et al, 1994).

DIIb (amino acids 365 to 404) contains a single disulfide bond (C\(^{372}\) - C\(^{379}\)) and has no known function other than the requirement of its last four residues for full toxicity. It has been proposed that DIIb may participate in internalization by binding the receptor due to its close approximation to DIIa in the toxin’s fully folded tertiary structure and that it may contain information within the sequence to facilitate secretion (Siegall et al, 1989).

DIII (amino acids 405 to 613) contains the enzymatic site and a C-terminal endoplasmic reticulum retention and retrieval signal consisting of the amino acids REDLK. The C-terminal lysine (K) must be excised for intoxication, reportedly through carboxypeptidases in the blood plasma, which yield a REDL C-terminal sequence (Hessler and Kreitan, 1997). This signal closely resembles that of the mammalian signal KDEL through which the KDEL receptor (KDEL-R) recognizes proteins that have escaped the golgi apparatus and returns them to the endoplasmic reticulum (ER) (Weldon and Pastan, 2011). Studies have shown that replacing ToxA’s native REDLK signal with the mammalian KDEL signal enhances KDEL-R receptor binding leading to a several fold increase in toxicity (Kreitman and Pastan, 1995). Upon entering the ER, the remaining toxin fragment then moves into the cytosol where it is responsible for the ADP-ribosylation of the eukaryotic elongation factor 2 (eEF2). Ribosylation occurs at a post-translationally modified histidine residue on eEF2, known as dipthamide, which causes inhibition of the eukaryotic translational machinery. Wild type (WT) ToxA and chimeras of ToxA are known to induce apoptosis in toxin sensitive cancer cell lines.
through diminished expression of the anti-apoptotic protein Mcl-1 that leads to activation of Bak, which in turn disrupts mitochondrial membrane stability (Du et al, 2011 and Andersson et al., 2004). Mitochondrial permeability and the release of the pro-apoptotic mediators within the mitochondria then trigger apoptosis by activating the caspase pathway (Jenkins et al., 2004).

**Chimeras of ToxA**

Therapeutic applications of WT toxins such as ToxA are essentially non-specific and can lead to inadvertent toxicity to non-diseased tissue. Therefore, bacterial vectors with the ability to express these toxins pose a potential risk since localization within off-target tissues have been reported with tumor targeted-*Salmonella* (Clairmont et al., 2000). To address this issue, the use of a hypoxia driven promoter, requiring *Salmonella* to localize and accumulate within hypoxic tumor tissues for gene induction, has been utilized to express and deliver the bacterial cytolysin, HlyE (Ryan et al., 2009).

Employing dual expression systems, which contain the genes for the bacterial toxin cytolysin A (ClyA) and the luciferase reporters renilla luciferase (rluc) or firefly luciferase (fluc) for the visualization of bacterial distribution, ensure that toxin expression is exogenously induced at the appropriate time as demonstrated in mouse models (Jiang et al., 2013). These applications provide innovative approaches that may increase the safety profile of vector delivery of WT toxins.

Alternatively, delivery of targeted chimeric-toxins by a tumor targeted-*Salmonella*, specific to receptors overexpressed by tumor cells, may eliminate undesired
host toxicity by providing an intrinsic secondary targeting mechanism (Quintero et al., 2016). Chimeras of ToxA have been experimentally created by truncating the binding domain (DIa), forming a 40 kDa C-terminal fragment known as PE40 (Figure 1 center panel), that was chemically or genetically fused to ligands specific for receptors over-expressed by cancer cells (Weldon and Pastan, 2011). Ligand based fusions have included interleukin 2 (IL-2), interleukin 6 (IL-6) and transforming growth factor-alpha (TGFα), which bind an internalizing receptor and lead to cell specific cytotoxicity (Lorberboum-Galski et al., 1988, Siegall et al., 1988, and Chaudhary et al., 1987).

Fusions of TGFα to PE40 (Figure 1, lower panel) have been extensively studied in E. coli. When an N-terminal secretion signal derived from the E. coli outer membrane porin A (OmpA) was fused to the N-terminal sequence of TGFα:PE40, soluble samples taken from the periplasm and extracellular milieu were shown to have activity against tumor cells overexpressing the TGFα cognate receptor, epidermal growth factor receptor (EGFR) (Siegal et al, 1989). The OmpA signal peptide is recognized by the Sec dependent pathway to direct proteins harboring it into the oxidizing periplasmic compartment, where disulfide bond isomerases (Dsb’s) may enhance the folding efficiency of proteins containing cysteines that must oxidized to form disulfide bonds. Importantly, full processing of TGFα in mammalian cells results in the active mature peptide (MW ~5.5 kDa) comprised of only fifty amino acids, yet contains six cysteine residues forming three disulfide bonds in a Cys⁸-Cys²¹, Cys¹⁶-Cys³², and Cys³⁴-Cys⁴³ convoluted folding pattern (McInnes et al., 1998). Thus, the activity of TGFα:PE40 was improved nearly ten-fold when the chimera was unfolded and refolded chemically, when
taken from the periplasm or inclusion bodies, due to enhanced folding efficiencies which the bacteria themselves could not achieve.

Genetically substituting the cysteines within PE40 to alanines in a fusion known as TP-40, facilitated the chemical folding process and further increased receptor binding with a slight decrease in cell toxicity, though the proteins formed inclusion bodies and were harvested from whole cell lysates (Edwards et al., 1989). Deletion of DIb from PE40 yields a 38 kDa fragment known as PE38, which was shown to have increased activity over PE40 when taken from the periplasm or chemically folded when derived from inclusion bodies (Kreitman et al., 1992). The increase in activity was attributed to the removal of the two cysteines within DIb that formed a disulfide bond, which decreased the complexity of PE38 while improving folding efficiency and enhancing both binding activity and toxicity.

One of the most toxic chimeras lacked a signal peptide and was completely harvested from inclusion bodies. It contained an N-terminal 35 kDa ToxA fragment, a downstream fusion with TGFα, followed by the C-terminal mammalian ER retention signal KDEL, and was known as PE35/TGFα-KDEL (Seetharam et al., 1991). The smallest chimera, TGFα-GIII-KDEL (MW ~31 kDa), completely lacked a signal peptide and DII and had the ligand and toxin domains separated via the short peptide linker GGGGS between domains, which provided spacing, flexibility and enhanced folding (Kihara and Pastan, 1994). The addition of the linker increased the activity of the naturally folded protein taken from the periplasm nearly 700-fold. Interestingly, without a secretion signal this chimera was found in the periplasm in a soluble form and was toxic.
to cells though it lacked the translocation domain (DII), indicating that both the secretory path(s) and mechanism(s) of toxicity are incompletely understood.

**Epidermal growth factor receptor (EGFR)**

TGFα belongs to the epidermal growth factor (EGF) – like family of peptides that bind to the epidermal growth factor receptor (EGFR) leading to increased cell growth and differentiation in normal mammalian physiology. Overexpression of EGFR is seen in many cancers of the breast, colon, lungs and brain, and its activation causes upregulation of signaling pathways that increase proliferation rates, anti-apoptotic mechanisms and metastatic properties (Simon and Fitzgerald, 2016). The extracellular binding domain of EGFR is composed of four domains (DI, DII, DIII, and DIV) and requires ligand binding followed by receptor coupling for signal transduction to occur (Yewale et al., 2013). Activation of EGFR via interaction of its ligand through DI and DIII exposes the dimerization arm in DII allowing the coupling with a second activated receptor. Receptor dimer formation leads to activation of the cytosolic tyrosine kinase domains through phosphorylation, subsequently triggering proliferative signal transduction pathways, including Ras/MAPK, Src, STAT 3/5, PI3 kinase and others.

Due to the abnormal overexpression of EGFR and its function in tumor cell proliferation and metastasis, EGFR-targeting in cancer therapy has been widely explored (Yewale, 2013). Monoclonal antibodies (mAb) and tyrosine kinase inhibitors (TKI) against EGFR have been approved for therapeutic use by the United States Food and Drug Administration (FDA), while several other targeted therapeutics continuously are
being developed and studied. Cetuximab was the first monoclonal antibody approved for
cancer treatment targeting EGFR. It is a mouse/human chimeric monoclonal antibody
that competitively inhibits EGFR signaling through blocking the ligand-binding region
and has been approved for head/neck cancers or metastatic colorectal cancers in
combination with irinotecan (Mehra et al., 2008 and Cunningham et al., 2004). Erlotinib
(aka CP-385,774) is a chemical based tyrosine kinase inhibitor that prevents auto-
phosphorylation of EGFR, arresting cell cycle progression at the G1 phase (Moyer et al.,
1997). It was the first approved anti-cancer drug of its kind and is the first-line treatment
for non-small cell lung cancers harboring EGFR mutations (Nguyen and Neal, 2012).

Targeting EGFR with chimeric toxins is dependent on the ability of the
receptor/ligand complex to become internalized through which the toxin may gain entry
into the cytosol. Internalization of EGFR is mediated by coupling of the receptor and the
formation of clathrin-coated pits (CPP) causing invagination of the membrane, forming
endocytic vesicles that may or may not fuse with lysosomes (Waterman and Yarden,
2001). Lysosomal fusions lead to ubiquitination of the receptor and degradation by
proteasomes, while other non-fused receptor containing vesicles are recycled to the cell
surface or, as in the case of ToxA chimeras that have bound the receptor, directed to the
golgi apparatus through retrograde trafficking due to the presence of proteins containing
an ER retention and retrieval signal.
Expression of ToxA chimeras by tumor-targeted Salmonella

In order for tumor selective Salmonella to deliver a targeted toxin, the chimera must be engineered such that the bacteria themselves can synthesize a product that is soluble, directed into the periplasmic space, and is secreted or released into the extracellular environment with good cytotoxic activity. An extensive literature search was conducted to select representative TGFα:ToxA chimeras that were shown to accumulate in the periplasm in a soluble and active form. Of the various TGFα:ToxA genetic constructs, three TGFα:ToxA chimeras were cloned and expressed in slightly modified forms. (Edwards et al., 1989; Kihara and Pastan, 1994; Kreitman et al., 1992). All forms include a fusion of TGFα upstream of DIII of ToxA. Components also include the Type II secretion signal derived from OmpA, a novel addition of a long flexible linker (GGGGS)₃, referred to as 3GS and the presence or absence of Dlb. Chimeras containing a substitution of WT ToxA’s native C-terminal REDLK endoplasmic reticulum retention signal with the KDEL consensus sequence were also constructed. Co-expression of the pColE3 lysis protein, which activates outer-membrane porins and phospholipase activity that causes an increase in outer membrane permeability, was utilized to facilitate secretion into the extracellular milieu (Morales et al., 2014 and Carward, 2004). A comparison of the cytotoxicity of culture supernatants from VNP20009 expressing these chimeras was assessed utilizing tissue culture viability assays. The work described in this thesis describes the first genetic engineering of TGFα:ToxA chimeras that are secreted or release by Salmonella typhimurium str. VNP20009 and selectively kill EGFR over-expressing cancer cells.
MATERIALS AND METHODS

Fusion model of a TGFα:PE40 chimera

For a theoretical analysis of the proposed chimeras, a fusion model of EGFR targeting chimeric toxin TGFα:PE40 was created. Briefly, the 1IKQ.pdb file containing crystallography structure of Pseudomonas Exotoxin A (PBD ID: 1IKQ, Wedekind et al., 2001) obtained through X-ray crystallography and the 1YUF.pdb file containing the solution structure of recombinant human TGFα obtained through solution nuclear magnetic resonance (NMR) spectroscopy (PBD ID: 1YUF Moy et al., 1993) was downloaded from the RCSB Protein Data Bank (www.rcsb.org., Berman et al., 2002). The files were opened in Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) to generate a 3-dimensional structure Image 1IKQ (Exotoxin A) that was colored coded as follows: DIA Red, DII Yellow, DIB Purple, DIII Green (Figure 1, Upper Panel). Image 1YUF (TGFα) was colored blue. A custom program was created in Python (Python Software Foundation. Python Language Reference, version 2.7) by Dr. Stan Metzenberg (California State University, Northridge) that was used to open the .pdb files in a text format that aligns the atoms within the residues of the protein through their respective (x, y, z,) 3-dimensional coordinates. A new program was written in Python that formed an N-terminal truncation of DIA of Exotoxin A (1IKQ) through the numbered residues leaving a C-terminal fragment consisting of DII, DIB and DIII, known as PE40 (Figure 1, Center Panel). A second section of new written code fused the TGFα ligand (1YUF) to the N-terminus of PE40 and image generated using Pymol (Figure 1, Lower Panel). The complete written code in Python language is provided in the Appendix.
**Bacterial strains, media, and plasmids**

Bacterial strains are listed in Table 1. All strains were stored frozen at 80°C in 15% glycerol. Bacterial media used were Luria Bertani (LB; 1% tryptone, 0.5% yeast extract, and 1% NaCl) broth or LB plates containing 1.5% agar (Miller, 1992), CTY (0.2% casamino acids, 0.2% tryptone, 0.1% yeast, and 0.9% NaCl adjusted to pH 7.4 with 0.2 M NaOH) (described further below), tryptic soy broth (Accumedia, Baltimore, MD), Mueller Hinton broth (Oxoid LTD., Basingstoke, Hampshire, England) and M56 minimal media. Ampicillin was used at a concentration of 100 μg/mL (Amp100) except in CTY which used at 50 μg/mL (Amp50). The expression plasmid utilized in this work is derived from pTrc99a (Amann et al., 1988; GenBank U13872.1) where the trc promoter and lacIq were removed with the enzymes NdeI and NcoI and replaced with the arabinose-inducible operon derived from the plasmid pBAD-IEE (GenBank AB598835) based on a previous study in *Salmonella* (Lossner et al., 2007). This plasmid, named pAra99a (pAra-1), lacks araC and therefore relies on diffusion of the chromosomal gene product AraC for repression.

**Development of the novel bacteriological media CTY**

The constituents of LB (1% tryptone, 0.5% yeast, 1% NaCl) were tested individually to determine the toxicity of each component. 10 μL of each sample was delivered to the MDA-MB-468 breast cancer cell line that were seeded in 96 well tissue culture plate. TSB, MH broth, M56 minimal media, M56 minimal media supplemented with 0.4% casamino acids and its constituents (M56 minimal media or 0.4% casamino acids) were
also evaluated for their potential as alternative growth media. PBS acted as a second control, which is commonly used in tissue culture and is generally well tolerated by mammalian cells. A methylthiazol diphenyltetrazolium (MTT) cell viability assay (described below) was conducted and the raw data was normalized to a no treatment control after subtracting background. A novel media, CTY was developed based on the results of the media tests. CTY Amp₅₀ was inoculated with a freshly streaked colony and allowed to incubate overnight at 37°C with rotation at 15 RPM. The bacterial culture was then sub-cultured in fresh CTY Amp₅₀ at a 1:5 dilution and allowed to incubate for 2 hours with rotation. The cultures (O.D. ~ 0.5) were then induced with 0.1% arabinose and incubated for 16 hours with rotation to allow for the accumulation of the proteins of interest within the media. The active supernatants were harvested by centrifugation at 3000 x G at room temperature for 10 minutes to pellet the bacteria and then the supernatant sterilized by passage through a low protein binding 0.2 µM polyethersulfone (PES) filter (Whatman, Buckinghamshire, UK; Puradisc 25 AS). The sterile supernatants were used on the same day they were prepared for cytotoxicity and enzyme activity assays. Culture supernatants derived for *Salmonella* str. VNP20009 harboring an empty plasmid vector were assessed for toxicity along with PBS against MD-MB-468 breast adenocarcinoma cells. The data from the MTT viability assays were normalized to a no treatment control after subtracting background and a Student’s t-test was used for statistical analysis. CTY Amp₅₀ was utilized as the sole bacterial growth media along with the induction protocol described above to assess for bacterial secretion or release of chimeric toxins in all subsequent viability and enzyme assays.
Preparation of electro-competent bacteria using a plate method

Electro-competent cells were made by the method of Enderle and Farwell, 1998, that was modified to improve competency, cell density, and volume. LB-0 solid growth media (LB lacking NaCl; Murray et al., 2001) was found to improve transformation by eliminating excess ions in the media. Freshly streaked bacteria that were incubated overnight at 37°C were re-streaked on a fresh plate and allowed to incubate overnight (~16 hours) at room temperature. The following day 1 mL of sterile ice cold nanopure water was added directly to the plate, the cells loosened with sterile a loop, followed by harvesting the cell suspension using a pipette. The bacteria were then spun down for 30 seconds at 10k x G and the supernatant discarded. The pellet was re-suspended and washed in 500 µL of sterile ice cold nanopure water and repeated for a total of five washes. The final pellet was then re-suspended with 10% glycerol at ten times the pellet volume, aliquoted and stored at -80°C.

PCR and cloning

Oligonucleotide primers used to construct the TGFα:ToxA chimeras are listed in Table 2. High-fidelity polymerases were utilized according to manufacturer’s recommendations (Phusion Hot Start II, Fermentas/Thermo Fisher, Waltham, MA); Phusion, Finnzymes/Thermo Fisher; KOD Hot Start Master Mix, Novagen/EMD Millipore, Billerica, MA). The PCR program consisted of one cycle of 98°C (Phusion) or 95°C (KOD) for 2 minutes followed by 35 to 40 cycles of 95 or 98°C for 10 seconds, 58°C for 30 seconds and 72 (Phusion) or 70°C (KOD) for 15–30 s/Kb, with a final extension of 5 minutes.
Variation to annealing temperatures are noted in Table 2. The PCR products or digested plasmid vectors were loaded onto a 0.9% or 1.2% agarose gel (depending on fragment size) in tris-acetate ethylenediaminetetraacetic acid EDTA (TAE: 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA), electrophoresed at 120V, then visualized using ethidium bromide under 360 nm wavelength ultraviolet light. The corresponding band was cut out with a clean razor blade, frozen, then sliced and passed through 0.45um sterile filter (Merck Millipore, Cork, Ire., Ultrafree MC 0.45 mM PVDF membrane cartridge). The DNA in the eluent was precipitated using 3M sodium acetate and ice cold ethanol and allowed to dry, then resuspended in sterile nanopure water. Fast digest restriction enzymes (Fermentas) were utilized in all cloning steps, with plasmid vector digests co-incubated with shrimp alkaline phosphatase (Fermentas). Ligations utilized T4 ligase (Fermentas) with an overnight incubation at 16°C and were transformed into chemical competent E. coli str. EC100 (Table 1). Successful transformants were screened by restriction digest and sequences confirmed by DNA sequencing of both strands (Sequetech, Mountain View, CA) using sequencing primers listed on Table 3. Sequence analysis was conducted using 4Peaks software (Nucelobyes, Aalsmeer, Netherlands) and plasmid maps and chimeric sequence graphics were created in Geneious (Biomatters, Auckland, NZ). After confirming sequences, the plasmids were transformed into electro-competent strains listed in Table 1. For Salmonella strains, electro-competent JR501 was initially transformed to provide methylation patterns, followed by mini-prep and transformation into electro-competent VNP20009.
Overlapping PCRs

Overlapping PCRs (Hoover and Lubkowski, 2001 and Stemmer et al, 1995) were conducted using initial independent PCR reactions for each component in the chimeric sequence (see Table 1 for PCR template), which were purified as indicated above. Approximate equimolar concentrations of each component was mixed into the 1st round overlapping PCR for a total of 10 cycles. 2.5 µL of the 1st round PCR was then introduced into a 2nd round PCR (50 µL total reaction volumes, 40 cycles) with the outer most primers as indicated in Table 2. Oligonucleotide synthesis (DNAworks; Hoover and Lubkowski, 2001) overlapping PCR reactions were performed similarly, except there were no initial independent PCR’s. Overlapping PCR products were then purified through gel electrophoresis, digested and cloned into their respective vectors.

Cloning of wild type ToxA and the pColE3 lysis protein

The wild type (WT) ToxA sequence was amplified using the Pseudomonas aeruginosa str. PA103-LAC in a colony PCR. Briefly, a single colony from a freshly streaked plated was suspended in 40 µL of sterile nanopure water and 1 µL of the suspension used as a template in PCR. The PCR product was digested and cloned into pAra-1 to create pDQ14 (Table 2). Amplification of the pColE3 lysis protein sequence including its Shine Delgarno sequence (bases 2660–2815, Morales et al., 2014; GenBank KM287568) utilized pColE3 as the template. The PCR product was digested and cloned into pAra-1 to create pDQ46. Templates, primers, annealing temperatures, restriction enzymes and inserted sequences are indicated in Table 2.
Gene synthesis and cloning of TGFα:ToxA chimeras

The general construction scheme of the TGFα:ToxA chimeras is shown in Table 2, consisting of a secretion signal sequence, targeting ligand, presence or absence of a flexible linker, and toxin domain made modular through the use of unique restriction sites. Chimeras were constructed through various overlapping PCR’s and sub-cloning steps with the template, primer sequences and restriction enzymes indicated in Table 2. Inserted synthetic gene fusions are also indicated in the table for reference. Initially, the products of three independent PCRs that incorporated a fusion of the WT ToxA secretion signal (ToxA SS) to TGFα, amplification of domain II of ToxA with a 25 amino acid deletion (DII (Δ25aa)), and amplification of domain Ib (DIb) and III (DIII) were mixed in an overlapping PCR to create a chimeric sequence that was cloned into pAra-1 using NcoI/BspHI and XbaI and designated pDQ28. pDQ28 was modified to include two glutamates in tandem downstream of the ToxA SS and a substitution of the C-terminal consensus sequence KDEL for the naturally occurring REDLK that was generated through PCR and inserted via SmaI and XbaI, which generated pDQ50. A template-less oligonucleotide synthesis (DNAworks; Hoover and Lubkowski, 2001) PCR was utilized to construct the Salmonella codon optimized (Codon Optimization Tool, Intergrated DNA Technologies) OmpA SS that was cloned into pDQ50 to create pDQ53, using the enzymes SacI and PvuII. The long flexible linker (GGGGS)₃ or 3GS was codon optimized and created through oligonucleotide synthesis and blunt-end cloned into pDQ53 by digesting the vector with SmaI only and the PCR product with EcoRV and SmaI, which yielded pDQ77.
Construction of OmpA:TGFα:3GS:DIb:DIIIK (O-T-G-DlbK) and OmpA:TGFα:3GS:DIb:DIIIK (O-T-G-DlbR)

DIb (excluding its first 4 amino acids) and DIIIK were amplified and cloned into pDQ77 with SmaI and XbaI to create an in frame synthetic gene fusion consisting of the OmpA secretion signal, the TGFα ligand, a long flexible linker, and a truncated ToxA fragment containing domains DIb and DIII with a C-terminal KDEL consensus sequence and forms the genetic sequence OmpA:TGFα:3GS:DIbK or O-T-G-DlbK. This plasmid was designated pDQ95. To compare chimeric ToxA fusions with WT ToxA, the removal of the KDEL consensus sequence and replacement with the WT ToxA sequence was required. This was achieved by subcloning a C-terminal fragment from pDQ14 using AatII and XbaI, which contains the WT ToxA sequence, into pDQ95 creating pDQ104 with the inserted chimeric sequence OmpA:TGFα:3GS:DIbR (O-T-G-DlbR) containing the C-terminal REDLK sequence.

Construction of OmpA:TGFα:3GS:DIIIK (O-T-G-DIIIK) and OmpA:TGFα:3GS:DIIIK (O-T-G-DIIIK)

The amplified product of DIII, which includes the last ten residues of DIb (required for activity) at the N-terminus and the C-terminal KDEL consensus sequence was inserted into pDQ95 using SmaI and XbaI to create pDQ99, which forms the synthetic gene sequence OmpA:TGFα:3GS:DIIIK (O-T-G-DIIIK). This sequence was also modified to include the WT ToxA C-terminal consensus sequence through sub-cloning the EheI and
XbaI fragment from pDQ14 to create pDQ156 harboring the chimeric sequence OmpA:TGFα:3GS:DIII (O-T-G-DIII).

**Construction of OmpA:TGFα:3GS:PE38K (O-T-G-PE38K) and OmpA:TGFα:3GS:PE38R (O-T-G-PE38R)**

An overlapping PCR was utilized to create a construct that included the entire DII sequence fused to a DIIbDIIIK sequence that had the region within DIIb containing a disulfide bond deleted. This fusion creates a 38 kDa protein known as PE38. The PCR product was cloned into pDQ95 with the enzymes SmaI and XbaI to create pDQ121 that includes the sequence OmpA:TGFα:3GS:PE38K (O-T-G-PE38K). A C-terminal modification to the KDEL consensus sequence for the WT ToxA sequence REDLK that creates OmpA:TGFα:3GS:PE38R (O-T-G-PE38R) and designated pDQ155 was also constructed for comparison through subcloning of the EheI and XbaI fragment from pDQ14.

**Insertion of the pColE3 lysis protein sequence for co-expression.**

The pColE3 Lysis protein sequence and its native Shine-Delgarno sequence from pDQ46 was subcloned into the O-T-G-DIIbK, O-T-G-DIIIK, and O-T-G-PE38K containing plasmids with XbaI and PstI creating the poly-cistronic co-expression plasmids pDQ96, pDQ103, and pDQ122, respectively (Table 2).
Deletion analysis of O-T-G-PE38K

Constructs made for deletion analysis utilized PCR, oligonucleotide synthesis PCR, and sub-cloning with the template, primer sequences, plasmids and restriction enzymes indicated in Table 2. Deletion of the N-terminal OmpA SS was performed by an oligonucleotide synthesis PCR that was cloned into pDQ121 using NcoI and SmaI. This plasmid, pDQ145, contains the inserted synthetic gene sequence TGFα:3GS:PE38K (ΔOmpA). The binding ligand, TGFα, was also deleted through oligonucleotide synthesis and the PCR product cloned into pDQ121 that used SacI and SpeI to create pDQ144, which contains the sequence OmpA:3GS:PE38K (ΔTGFα). Deletion of the flexible linker, 3GS, was constructed by subcloning the PE38K containing fragment from pDQ121 into pDQ53, that contains the N-terminal fusion of OmpA:TGFα without a linker with the enzymes SmaI and XbaI, to create pDQ146 harboring the genetic sequence OmpA:TGFα:PE38K (Δ3GS). Inactivation of ADP-ribosylation activity was accomplished through a single amino acid substitution of glutamate for aspartic acid at amino acid position 553 of WT ToxA. This mutation was carried out though PCR and cloning into PDQ121 with EheI and XbaI to create pDQ127, which harbors the genetic construct OmpA:TGFα:3GS:PE38K-E553D (E553).

Tumor cells

The high EGFR expressing human breast adenocarcinoma cell line MDA-MB-468 (EGFR; Xu et al., 2005) was provided by Dr. Jonathan Kelber (California State University, Northridge). The low level EGFR expressing human cervical adenocarcinoma
cell line HeLa (Eiblmaier et al., 2008) was obtained from the ATCC. The H460 human large cell lung carcinoma that has undetectable EGFR expression (Kirk et al., 1994) was obtained from the National Cancer Institute Division of Cancer Treatment and Diagnosis (DCTD; Frederick, MD). All three cell lines were authenticated at the University of Arizona Genetics Core (Tucson, AZ). MDA-MB-468 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM), HeLa cells were cultured in Minimal Essential Media (MEM), and H460 were cultured in Roswell Park Memorial Institute medium (RPMI). All culture growth media were supplemented with heat-inactivated fetal bovine serum (10% vol/vol; Gibco/Life Technologies, Grand Island, NY) and 1% of penicillin-streptomycin (10,000 units penicillin and 10 mg/mL streptomycin; SigmaAldrich, St. Louis, MO) and cells incubated in the presence of 5% CO₂. Cells were passed or seeded using trypsin (0.5% trypsin 0.2% EDTA, Sigma –Aldrich) or harvested with a cell scraper for immunoblots.

**Cell proliferation and cytotoxicity assays**

To quantify the relative viability of cells treated with bacterial supernatants, phosphate buffered saline (PBS), or bacterial media, the methylthiazol diphenyltetrazolium assay (MTT; Calbiochem, San Diego, CA) that is based on the presence of functional cytosolic NAD(P) oxidoreductase enzymes (Mosmann, 1983) was utilized. Briefly, live cells reduce the MTT substrate, which leads to the formation of insoluble purple-formazan crystals that are solubilized with an organic solvent such as dimethylsulfoxide (DMSO), then spectrophotometrically quantified at A₅₇₀ (Spectramax M3, Molecular Devices;
Sunnyvale, CA). Cell lines were seeded at 2,500 cells/100 µL/well in a 96-well culture plate and allowed to adhere overnight. The following day, 10 µL of undiluted or diluted (2-fold in PBS) bacterial supernatants were added to the tissue culture plates and incubated for 72 hours. The MTT reagent was then added to the cells, developed and absorbance taken. The results were normalized using a PBS control (100% survival) after subtracting the background from a media only control using Excel and Prism 6.0f (Graphpad, La Jolla, CA). Assays were done in triplicate to account for operator error. Each assay was repeated at least three times on separate days except where noted. The data for individual treatment groups were pooled and the log of the volume delivered plotted on a graph with their respective mean and standard deviations (SD).

Approximation (~) of the volume required to achieve 50% inhibition (IC₅₀) was calculated using an arithmetic ratio for treatment groups that approached but did not achieve an IC₅₀. Actual IC₅₀ values were determined by pooling the mean of each independent experiment and plotting it using sigmoidal, 4PL, log function in Prism, which generates IC₅₀ values and their 95% confidence intervals (CI), and Log IC₅₀ values and their standard error. A one-way ANOVA (P < 0.05) followed by post hoc Tukey’s multiple comparison test was used for statistical analysis of Log IC₅₀ values. An IC₅₀ bar graph indicating 95% CI (error bars) was utilized to graphically compare activity of the ToxA chimeras. Actual and logarithmic supernatant volumes are indicated on their respective axes.
**Protein detection and quantification**

Purified *Pseudomonas* ToxA (Sigma Aldrich) was quantified using an extinction coefficient E₁% = 11.9 with A₂₈₀ measurement (NanoDrop 2000 s, Thermo Fisher) and was used as a standard in western blots. Serial dilutions of the ToxA standard and 10 µL of VNP20009 produced ToxA supernatants were loaded onto SDS-PAGE with a 10% resolving gel. Proteins were transferred onto a nitrocellulose membrane (overnight at 34 volts at 4°C) and blocked with 3% bovine serum albumin in T-TBS washing buffer (0.05% Tween 20, 50 mM Tris Base, 0.5 M NaCl, pH 7.4) for 30 minutes, followed by overnight incubation at 4°C with a rabbit anti-*Pseudomonas* ToxA polyclonal antibody (Sigma –Aldrich) diluted 1:40,000 in blocking buffer. The primary antibody was washed three times, then a goat anti-rabbit alkaline phosphatase secondary antibody (Jackson Laboratories, West Grove, PA) diluted 1:40,000 in blocking buffer or goat anti-rabbit horse radish peroxidase diluted 1:20,000 (Jackson Laboratories, West Grove, PA) were added and incubated for 1 hour. The wash step was repeated and the protein detected by overnight incubation with the alkaline phosphatase colorimetric substrate and enhancer, 5-bromo-4-chloro-3’-indolyphosphate BCIP [stock solution: 50 mg/mL in 100% DMF] and nitro-blue tetrazolium (NBT) [stock solution: 50 mg/mL in 70% dimethyl formamide (DMF)] that were diluted in alkaline phosphatase buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl₂, pH 9.0) 1:150 and 1:300, respectively as a mixture. Horse radish peroxidase secondary antibody was detected by enhanced chemi-luminescence (ECL) and film (Thermo-Fisher). The ToxA standards were quantified using Image J (Schneider et al., 2012) to construct a standard curve, with the VNP20009 produced ToxA analyzed by linear regression using Excel (data not shown). ToxA chimeras were blotted similarly.
or utilizing a secondary antibody conjugated with a horse radish peroxidase that was detected by enhanced chemi-luminescence (ECL) and film (Thermo-Fisher). Since the TGFα:ToxA chimeras have varying ToxA antigens, due to the different levels of WT ToxA truncations present in each fusion, quantification with the WT ToxA polyclonal antibody is inadequate. Therefore, quantification of the chimeras was accomplished via the N-terminal ligand TGFα, which is common to all three constructs. Purified human recombinant TGFα (hrTGFα; R&D Systems, Minneapolis, MN) was quantified by using recombinant human insulin (Santa Cruz Biotechnology, Dallas, TX) as a standard in the 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) protein assay with 0.1% Triton X-100 added to enhance efficiency (Molecular Probes, Eugene, OR). CBQCA is fluorescent assay useful for quantifying small peptides in solution through interaction of free amines. Samples were loaded onto a 96 well black bottom plate and fluorescence quantified through absorbance/emission of 465/550 nm then plotted using linear regression. Serial dilutions of quantified TGFα (2.0 µg, 0.67 µg, 0.20 µg and 0.067 µg) were loaded along with VNP20009 supernatant samples (200 µL) that were concentrated by pyrogallol red molybdate methanol (PRMM) (Caldwell and Lattemann, 2004). SDS-PAGE with a 15% resolving gel was utilized to separate the proteins, which were then transferred onto a nitrocellulose membrane as indicated above. The blot was incubated overnight at 4°C with mouse anti-TGFα monoclonal antibody (Clone MF9; Abnova, Taipei, Taiwan) that was diluted 1:200 in blocking buffer and a secondary rabbit anti-mouse alkaline phosphatase antibody (Jackson Laboratories) that was diluted 1:40,000 were used to detect the protein. To determine the expression level of EGFR among the cancer cell lines, the cells harvested by scraping were washed three times in ice cold
PBS, which were centrifuged at 1,000 x G for 5 mins at each wash step. The cells were lysed using ice-cold NP-40 buffer (1.0% NP-40, 150 mM NaCl, 50 mM Tris, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA, incubated on ice and vortexed every few minutes for 30 mins. The cell lysate was then centrifuge to remove insoluble material and nucleic acids, and the protein was quantified using the bicinchoninic acid (BCA) assay (Pierce/ Thermo Fisher) that is compatible with detergents. 20 µg of each tumor cell lysate was loaded on to a SDS-PAGE with a 10% resolving gel. A rabbit polyclonal EGFR antibody (Rockland, Gilbertsville, PA) diluted 1:1,000 and a goat anti-rabbit horse radish peroxidase antibody (Jackson Laboratories) diluted 1:20,000 were used to detect the receptor by enhanced chemi-luminescence (ECL) and film (Thermo-Fisher). As a loading control, a blot was probed for the house keeping protein, glyceraldehyde 3-phosphate dehydrogenase (GADPH) using a rabbit anti-GADPH primary (Thermo Fisher) diluted 1:2,500 and the horseradish peroxidase secondary using the ECL substrate and film.

**JC-10 mitochondrial membrane depolarization assay**

Induction of apoptosis was determined by quantifying mitochondrial polarity using the fluorescent fluorophore, JC-10 (Enzo Life Sciences, Farmingdale, NY). Mitochondria are normally in a polarized state with a negatively charged inner membrane potential. JC-10 is a lipophilic, cationic dye that is highly permeable to mammalian cells and selectively diffuses from the cytosol into the mitochondria. JC-10 forms aggregates in healthy mitochondria and can be quantified through fluorescence. Depolarization or loss of membrane potential is an early indicator of cells undergoing apoptosis, which prevents
aggregate formation within the mitochondria leading to accumulation of the dye within the cytosolic compartment in its monomeric form (Patel et al., 2016). For the mitochondrial depolarization assay, cells were seeded at 5,000 cells/100 μL/well in a 96 well black bottom plate (Corning, Corning, NY). The cells were allowed to adhere for 24 hours, then were treated with 10 μL of induced VNP20009 supernatant for 18 or 22 hours. The media from the treated cells was then aspirated and the cells stained with 2 μM JC-10 in cell culture media (100 μL total volume) for 15 minutes. The JC-10 was aspirated and stained with 1 μg/mL Hoechst in PBS (100 μL total volume) for 10 minutes, followed by aspiration and addition of 100 μL PBS. Total relative JC-10 (aggregate) fluorescence units at 528/590 nm (Ex/Em) was divided by total Hoechst (nuclear) fluorescence staining at 340/460 nm (Ex/Em), and normalized to the empty vector control to determine relative percent fluorescence/cell.

Detection of bacterial outer-membrane disruption using a phosphatase assay

Release of phosphatases normally located within the periplasmic compartment of Salmonella (Kier et al, 1977) were used as an indicator of bacterial stress. Bacterial membrane disruption was qualitatively measured using the phosphatase substrate p-nitrophenol phosphate (p-NPP) (Sigma Aldrich). 75 μL of bacterial supernatant was mixed with an equal volume of phosphatase buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl₂, pH 9.0) in a 96 well black bottom plate, followed by the addition of the p-NPP substrate (1.4 mg/mL final concentration) to initiate the reaction. The reaction mixture was then incubated in the dark at room temperature for 2 hours followed by an
absorbance read at 405 nm. The raw data was normalized to an empty vector control after subtracting background and plotted in Prism.
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<th>Strain</th>
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<td>EC100</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ&lt;sup&gt;−&lt;/sup&gt; rpsL&lt;sup&gt;−&lt;/sup&gt; (Str&lt;sup&gt;R&lt;/sup&gt;) nupG</td>
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<td><em>Salmonella</em> Genetic Stock Center, Calgary, Canada, strain 1593; Tsai et al., 1989</td>
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Table 2. Cloning schematic, Plasmids, primers, and restriction enzymes

**Cloning Schematic**

![Cloning Schematic Diagram](image)

General chimeric DNA sequence schematic. The schematic indicates the relative positions of the DNA sequences encoding each functional domain within the chimera. DNA restriction sites are indicated with red arrows and demonstrate the modularity of the construct. The dotted line indicates the position of insertion of the flexible linker utilizing the *SmaI* site.

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<th>Gene Insert/Gene Fusion</th>
<th>PCR Primers Forward (F), Reverse 1 DNA Works (P)</th>
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RESULTS

Theoretical model of a TGFα:PE40 chimera.

The results of the virtual fusion of TGFα and PE40 are shown in Figure 1. The binding domain (D1b) was deleted from the I1KQ generated image of Exotoxin A (Upper Panel) resulting in the 40 kDa fragment known as PE40 (Center Panel). The TGFα image (1YUF) was merged to PE40 to create the virtual chimeric protein TGFα:PE40 (Bottom Panel). The results indicate that the written Python program is useful in generating images in Pymol of fused proteins whose 3-dimensional structures have been solved through X-ray crystallography or solution NMR.
Figure 1. Hypothetical model of TGFα:PE40 chimera. (Upper panel) Image of 1IKQ (Exotoxin A) created with PyMOL (DIIa Red, DII Yellow, DIIb Purple, DIII Green). (Center Panel) Truncation of Domain Ia of Exotoxin A using image 1IKQ created through custom Python programming and PyMOL. (Lower Panel) Fusion of TGFα (blue, image 1YUF) to PE40 through custom Python programming and PyMOL.
Development of the novel bacteriological media CTY

Initial screening utilizing LB broth showed toxicity towards MDA-MB-468 cancer cells and therefore an alternative bacterial growth media was necessary to evaluate the toxicity of an active bacterial supernatant. The relative toxicity of the bacterial media on the MDA-MB-468 cell line are shown in Figure 2 (Top Panel). TSB and MH broth along with M56 minimal media supplemented with 0.4% casamino acids, M56 minimal media, and 0.4% casamino acids were tested along with the LB components to determine potential alternative bacterial growth media (Figure 2, Top Panel). The results of the bacterial media toxicity test indicate that the LB components tryptone and yeast extract reduced cell survivability approximately 50%, while 1% NaCl was less inhibitory to the cells than PBS. Both TSB and MH broth also reduced cell viability, but were narrowly less toxic than the tryptone and yeast. M56 minimal media supplemented with 0.4% casamino acids or M56 alone were also toxic to the cells. However, 0.4% casamino acids alone had less of an effect on the cells than PBS as seen in the 1% NaCl treatment of the cells. Based on these results, the novel bacteriological media, CTY (Casamino acids, Tryptone, Yeast), was developed and evaluated for toxicity through the use of culture supernatants from Salmonella str. VNP20009 harboring an empty vector grown in CTY media. As indicated in (Figure 2, Bottom Panel), the bacterial supernatant showed a slight, but insignificant reduction in cell viability relative to a PBS control. Based on these results CTY was used in subsequent studies.
Figure 2. Assessment of bacterial media components on mammalian cell survival. (Top panel) Relative toxicities towards MDA-MB-468 associated with bacterial media components phosphate buffered saline (PBS), 0.5% yeast, 1% tryptone, 1% NaCl, Tryptic Soy Broth (TSB), Mueller Hinton (MH), M56 minimal media and 0.4% casamino acids, M56 minimal media, 0.4% casamino acids (n = 2). (Bottom panel) Toxicity of VNP20009 supernatants harboring an empty vector plasmid grown in CTY with 0.1% arabinose induction, against MDA-MB-468 breast cancer. All data were normalized to a no treatment control and an unpaired t-test was utilized for statistics (n = 9). The MTT assay raw data used to construct the graphs was obtained in collaboration with Jamie Carrafa at CSUN.
Quantification of VNP20009 produced WT ToxA and relative production of TGFα:ToxA chimeras using an anti-ToxA antibody.

The chimeric constructs used for protein expression are compared with WT ToxA in Figure 3, (Left Panel). A ToxA standard (MW 66 kDa) was used to quantify VNP20009 produced ToxA66 kDa (10 µL supernatant) that yielded 39.9 ng/µl (not shown). Supernatants (200 µL) containing the TGFα:ToxA chimeras containing the C-terminal ER retention sequence (REDLK) were precipitated using the PRMM method. The predicted molecular weights of the TGFα:ToxA chimeras are 33 kDa for O-T-G-DIbR, 31 kDa for O-T-G-DIIIR and 44 kDa for O-T-G-PE38R. The results of the western blots show that all of the constructs, including the WT ToxA standard, ran ~5 kDa higher than their predicted molecular weights (Figure 3, Right Panel). This is presumably due to the use of pre-stained molecular weight ladder that has a dye conjugated to the protein standards and is generally used for approximation of protein size. The VNP20009 production of the TGFα:ToxA chimeras detected by the anti-ToxA immunoblot varied with O-T-G-DIbR having the highest level of detection followed by O-T-G-DIIIR, then O-T-G-PE38R (Figure 3, Right Panel). The precipitation of supernatants using PRMM produced protein pellets of different sizes for each respective construct, with an inverse relationship to that of the level of specific targeted toxin detected on the blot. This was evident through the appearance of break down products on the blots for O-T-G- DIIIR and O-T-G-PE38R, which had significantly larger pellets than O-T-G-DIbR and the empty vector control (data not shown), and may indicate cellular stress related to differences in the structural components of the chimeras (Figure 3, Right Panel).
**Figure 3. Comparison of the wild type ToxA and ToxA chimeras.** Left panel A) ToxA; the wildtype toxin, +/- the E553D substitution. B) O-T-G-DIbR, a chimera containing an OmpA secretion signal, TGFα targeting domain, a 3GS linker, ToxA DIb and DIII followed by REDLK. C) O-T-G-DIIIR, a chimera containing an OmpA secretion signal, TGFα targeting domain, a 3GS linker, truncated DIb, and ToxA DIII followed by REDLK. D) O-T-G-PE38R, a chimera containing an OmpA secretion signal, TGFα targeting domain, a 3GS linker, ToxA DII, partial DIb (Δ365-380 amino acids) and DIII followed by REDLK. **Right panel**, an immunoblot using anti-ToxA. Lane 1, molecular weight standard. Lane 2, pyrogallol red molybdate methanol (PRMM)-precipitated VNP20009 empty vector culture supernatant (200 μl). Lane 3, ToxA standard (350 ng). Lane 4, VNP20009 ToxA culture supernatant (10 μl). Lane 5, VNP20009 O-T-G-DibR pyrogallol red molybdate methanol (PRMM)-precipitated culture supernatant (200 μl). Lane 6, VNP20009 O-T-G-DIIIR PRMM-precipitated culture supernatant (200 μl). Lane 7, VNP20009 O-T-G-PE38R PRMM-precipitated culture supernatant (200 μl). The figure and legend from Quintero et al., 2016.
**Relative toxicities of the TGFα:ToxA chimeras.**

The relative toxicity of supernatants from VNP20009 expressing WT ToxA or TGFα:ToxA chimeras are shown in Figure 4. The supernatants containing WT ToxA were highly toxic to the MDA-MB-468 cell line. The single amino acid substitution, E553D, within the active site of WT ToxA completely eliminated its toxicity, which is consistent with previous reports (Douglas and Collier, 1987). Among the TGFα:ToxA chimeras, the supernatant with the highest toxicity came from the expression of O-T-G-PE38R, which was followed by the expression of O-T-G-DIbR, then O-T-G-DIIIR (Figure 4B).
Figure 4. Dose-response curves of wild type ToxA and ToxA chimeras. Dose responses were determined as relative percent survival of MDA-MB-468 breast adenocarcinoma cells following exposure to 2-fold dilutions of the respective VNP20009 culture supernatants. Panel A: ToxA wild type toxin (●) and ToxA with the E553D substitution (■). Panel B: The chimeric toxins O-T-G-DIbR (□); O-T-G-DIIIR (▲); and O-T-G-PE38R (○). The MTT assay raw data used to construct the graphs was obtained through collaboration with Jamie Carrafa and Lena Vincent at CSUN. The figure and legend are from Quintero et al., 2016.
Quantification of the TGFα:ToxA Chimeras.

The quantification of the TGFα:ToxA chimeras through antibody detection of TGFα are shown in Figure 5. The blot showed no reaction for PRMM precipitated supernatants of VNP20009 harboring an empty vector, demonstrating the antibody’s high specificity. The concentrations of targeted toxin in the culture supernatant, reported in ng/µL, and the volumes and concentration (µM) required to achieve a 50% inhibitory concentration (IC$_{50}$) are indicated in Figure 5B. Of the TGFα:ToxA chimeras, O-T-G-PE38R was the most potent (IC$_{50}$ = 0.3 µM), to the EGFR expressing cell line MDA-MB-468, though it produced the least amount of the targeted toxin. O-T-G-DIbR was detected at a higher concentration than O-T-G-PE38R and O-T-G-DIIIR, it was the least potent among the chimeras (IC$_{50}$ ~ 15.5 µM). The smallest chimera, O-T-G-DIIIR, had supernatants that were the least toxic, which were unable to achieve the 50% reduction seen with the other constructs. However, in terms of potency it was apparently second in order, behind O-T-G-PE38R with an IC$_{50}$ approaching 4.9 µM or greater. Since this analysis demonstrated that the activity O-T-G-PE38R was the most potent (IC$_{50}$ = 0.3 µM), it was selected for further modification and evaluation.
Figure 5. Quantification of the chimeric proteins present in culture supernatants and their relative cytotoxicities. **Top Panel:** Anti-TGFα immunoblot comparison of hrTGFα standards and PRMM-precipitated culture supernatants of O-T-G-DibR, O-T-G-DIIIR and O-T-G-PE38R. Lane 1, molecular weight standard. Lane 2, 2.0 µg hrTGFα. Lane 3, 0.67 µg hrTGFα. Lane 4, 0.20 µg hrTGFα. Lane 5, 0.067 µg hrTGFα. Lane 6, VNP20009 empty vector culture supernatant. Lane 7, O-T-G-DibR. Lane 8, O-T-G-DIIIR. Lane 9, O-T-G-PE38R. **Lower Panel:** IC₅₀ values of the TGFα:ToxA fusions present in the culture supernatants. The quantities of the fusion proteins present in the culture supernatants were calculated based on a standard curve of the hrTGFα bands in the top panel determined by densitometry using Image J. The volume of culture supernatants in µL/well required to achieve the 50% inhibitory values shown in Figure 2B were calculated from the interpolated dose response curves, which allowed the calculation of the IC₅₀ values in µM. The figure and legend are from Quintero et al., 2016.
IC₅₀ comparisons of the KDEL substitution and co-expression of the pColE3 lysis protein relative to WT ToxA and O-T-G-PE38R.

The IC₅₀ data comparing WT ToxA, O-T-G-PE38R, O-T-G-PE38K, and O-T-G-PE38K co-expressed with the pColE3 lysis protein are shown in Figure 6. The interpolated 4PL sigmoidal curves demonstrate the increase in toxicity attributed to the substitution of REDLK for KDEL with the co-expression of the pColE3 lysis protein further increasing toxicity (Figure 6A). The interpolated curve was used generate the IC₅₀ and Log IC₅₀ bar graph for comparison. The results show that supernatants harvested from the expression of O-T-G-PE38R required 1.42 µL to achieve an IC₅₀ (Figure 6B). Substitution of the native ER retention signal REDLK for the mammalian consensus sequence KDEL enhanced the toxicity of the chimera approximately 4-fold (IC₅₀ = 0.34 µL, P = 0.017) leading to a toxicity level greater than WT ToxA (IC₅₀ = 0.53 µL). Additionally, co-expression of the pColE3 lysis protein with the chimera containing the consensus sequence KDEL further enhanced its ability to kill MDA-MB-468 cancer cells (IC₅₀ 0.10 µL, P = 0.0049), which was approximately 5-fold greater than WT ToxA (P < 0.021).
Figure 6. Relative culture supernatant toxicities of wild type ToxA and chimeric variants. A) The individual dose response curves for the wild type toxA, O-T-G-PE38R, O-T-G-PE8K, and O-T-G-PE38K with the ColE3 Lysis. Error bars represent SD. Actual and logarithmic supernatant volumes are indicated on the x-axis. B) Comparision by volume (actual and log) of the supernatants required to achieve an IC50 against MDA-MB-468 tumor cells. The WT ToxA, O-T-G-PE38R, O-T-G-PE38K, and O-T-G-PE38K with the ColE3 lysis were compared by the quantity (in µL) of supernatant required to achieve an IC50 against MDA-MB-468 tumor cells. A one-way ANOVA followed by a post hoc Tukey’s test were utilized for statistics. Brackets indicate comparisons that were made and the level of significance (asterisks) as well as the actual P-value indicated below the bracket. The MTT assay raw data used to construct the graphs was obtained in collaboration with Jamie Carrafa and Lena Vincent at CSUN. The figure and legend from Quintero et al., 2016.
Deletion Analysis of O-T-G-PE38K.

The deletion analysis of the N-terminal components of O-T-G-PE38K and the active site mutation are shown in Figure 7. Deletion of OmpA secretion signal (ΔOmpA) from the expression construct prevented both the accumulation of the chimeric toxin in the culture supernatant (Figure 7A) and toxicity towards the cancer cells (Figure 7C). Deletion of the EGFR binding ligand (ΔTGFα) also led to complete loss of toxic activity, though the toxin was still detected in the culture supernatant due to the presence of the OmpA signal sequence, which is necessary for secretion. This is in agreement with previous studies that have shown accumulation of PE38 in the media when fused to the OmpA signal peptide (Kreitman et al., 1992). However, the amount of the toxin fragment detected was reduced relative to the complete construct O-T-G-PE38K, which may indicate a contribution of TGFα in the secretion process as described with other TGFα:ToxA chimeras that were found in the periplasm when no signal peptide was present (Kihara and Pastan, 1994). Removal of the long flexible linker (Δ3GS) led to a decrease in overall activity when compared to O-T-G-PE38K. The linker-less chimera was detected in the supernatant at a lower level than the complete O-T-G-PE38K. This difference may be due to improved solubility and/or the prevention of the degradation of the protein, which has been reported in other linker containing chimeras (Chen et al, 2013B). Although TGFα has been shown to induce apoptosis in MDA-MB-468 breast cancer cells at relatively high concentrations (Wang et al., 1997), the O-T-G-PE38K-E553D active site mutant indicates that TGFα was not contributing to the toxicity observed. The O-T-G-PE38K-E533D construct completely lost its toxic activity and was detected in the culture supernatant, demonstrating that the toxic activity of the unmodified O-T-G-PE38K was
provided by the ToxA fragment of the chimera and not TGFα. Co-expression of E3 lysis protein increased the cytotoxicity of the O-T-G-PE38K and correspondingly increased the amount of chimeric toxin detected through immunoblot.
Figure 7. Deletion analysis of the O-T-G-PE38K N-terminal components and comparisons with the E553D substitution and the co-expression of the ColE3 lysis protein. **Panel A:** ECL immunoblot against the ToxA fragment of each chimera used in the deletion analysis of O-T-G-PE38K. **Panel B:** Diagram of the parent O-T-G-PE38K and variants. **Panel C:** Dose responses from the constructs diagramed in the left panel were determined as relative percent survival of MDA-MB-468 breast adenocarcinoma cells following exposure to culture supernatants from VNP20009 expressing A) O-T-G-PE38K, B) ΔOmpA (O-T-G-PE38K with a deletion in OmpA), C) ΔTGFα (O-T-G-PE38K with a deletion in TGFα), D) Δ3GS (O-T-G-PE38K with a deletion of the 3GS linker), E) E553D (O-T-G-PE38K with a glutamic acid to aspartic acid substitution at the amino acid corresponding to number 553 of the wild type ToxA), n=2, and F) +ColE3 Lysis (O-T-G-PE38K co-expressed with the ColE3 lysis protein. The MTT assay raw data used to construct the survival graphs was obtained in collaboration with Jamie Carrafa and Lena Vincent at CSUN. The bottom panel of the figure and its corresponding text were taken from Quintero et al., 2016 with permission.
O-T-G-PE38K is selective for EGFR over-expressing cells.

The relative level of EGFR expression determined through immunoblot of cell lysates from the H460 lung, HeLa cervical, and MDA-MB-468 breast cancer cell lines are shown in Figure 8A, with the GADPH house keeping protein serving as a loading control. The results show a high level of EGFR expression in the MDA-MB-468 cells, low level EGFR in HeLa cells, and no expression in the H460 cell line. All cell lines were sensitive to supernatant from a *Salmonella* expressing WT ToxA at a relatively high degree. (Figure 8B, Center Panel). The cell lines treated with supernatant from a *Salmonella* expressing O-T-G-PE38K showed selectively toxicity to the high EGFR expressing cell line MDA-MB-468, with little to no activity against HeLa and H460, respectively (Figure 8C, lower panel). Mitochondrial staining using JC-10 and the nucleic acid stain Hoechst further confirmed the relative selectivity of O-T-G-PE38K. At 18 hours, the HeLa cells treated with ToxA showed a 55% reduction of the mitochondrial aggregation signal ($P = 0.0087$) indicating a depolarized state, while the cells treated with O-T-G-PE38K resulted in a hyperpolarized state relative to the empty vector control (Figure 9, Top Panel). Depolarization for MDA-MB-468 cell line was detected at 22 hours, for both the WT ToxA and O-T-G-PE38K treated cells with a 25% reduction ($P = 0.0014$) and 55% reduction ($P = 0.0001$), respectively, relative to the empty vector control (Figure 9, Lower panel). The differences in the amount of incubation time with the toxins required for the HeLa and MDA-MB-468 cells (18 hour vs 22 hour) is due to the increased sensitivity of HeLa to WT ToxA (See Figure 8B), which showed signs of toxicity as early as 8 hours (data not shown). Unlike the HeLa cells, MDA-MB-468 treated with WT ToxA required 22 hours to show signs of cellular stress and was more sensitive to O-T-
G-PE38K, which showed toxin sensitivity as early as 14 hours (data not shown). Taken together these results demonstrate the selective toxicity and apoptosis inducing ability of O-T-G-PE38K.
Figure 8. Relative expression levels of epidermal growth factor receptor (EGFR) in different tumor cell lines and the relative sensitivities of those cell lines to culture supernatants of VNP2009 expressing either WT ToxA or O-T-G-PE38K. A: Immunoblot of whole cancer cell lysates using antibodies against either human EGFR or human GAPDH. B: Dose-response curve of WT ToxA against H460 (●), HeLa (■) and MDA-MB-468 (▲) cancer cells. C: Dose-response curve of O-T-G-PE38K against H460 (○), HeLa (□) and MDA-MB-468 (△) cancer cells. The MTT assay raw data used to construct the graphs was obtained in collaboration with Jamie Carrafa and Lena Vincent at CSUN. The Figure and text were taken from Quintero et al., 2016 with permission.
Figure 9. JC-10 Mitochondrial depolarization assay. **Top Panel:** Relative fluorescence of the JC-10 stain for HeLa cells treated with supernatants from VNP20009 harboring an empty vector (EV) plasmid and expressing WT ToxA or O-T-G-PE38K developed at 18 hours. **Bottom Panel:** MDA-MB-468 cells treated in the same manner and developed at 22 hours. One-way ANOVA followed by a post hoc Tukey’s test were used for statistics.
Evaluation of the KDEL substitution and co-expression of pColE3 Lysis in the O-T-G-DIbR and O-T-G-DIIIR constructs.

The dose response curves for the KDEL substitutions and co-expression of the pColE3 lysis protein for the O-T-G-DIbR and O-T-G-DIIIR constructs are shown in Figure 10. Replacement of the C-terminal REDLK ER retention signal for the KDEL consensus sequence in O-T-G-DIbR, which produces O-T-G-DIbK, resulted in a bacterial supernatant that was more toxic to the MDA-MB-468 EGFR (Figure 10, Top Panel). Co-expression of the pColE3 lysis protein further increased toxicity, yielding a substantial gain versus O-T-G-DIbR expressed alone. This overall increase in toxicity did not reach the level of O-T-G-PE38R with the same genetic modifications as indicated by the results shown in Figure 10. However, the increase in activity due to co-expression of the pColE3 lysis protein with O-T-G-DIbK was approximately equal to the increase observed in O-T-G-PE38K with the same modification (Figure 10, Top and Bottom panel). Addition of the KDEL sequence to O-T-G-DIIIR and co-expression of the pColE3 lysis protein also increased overall toxicity of the supernatant, but it did not achieve the improvement in toxicity seen in the expression of the other constructs (Figure 10, Center Panel).
Figure 10. Effect of the addition of KDEL and pColE3 Lysis in O-T-G-DIbR and O-T-G-DIIIR. Supernatants from VNP20009 were exposed to MDA-MB-468 cells. (Upper Panel) Dose response curve of O-T-G-DIbR containing the C-terminal ER consensus sequence KDEL and its co-expression with ColE3 Lysis protein. (Center Panel) Dose response curve of O-T-G-DIIIR, O-T-G-DIIIK and O-T-G-DIIIK + E3 Lysis. (Bottom Panel) Dose response curve of O-T-G-PE38R, O-T-G-PE38K and O-T-G-PE38K + E3 Lysis. Error bars indicate SD (n = 2 for O-T-G-DIbK +/- E3 Lysis and O-T-G-DIIIK +/- E3 Lysis) The MTT assay raw data used to construct the graphs was obtained in collaboration with Jamie Carrafa at CSUN.
Detection of bacterial outer-membrane disruption using a phosphatase assay

The results of the phosphatase assay on supernatants from VNP20009 harboring an empty vector plasmid or encoding either WT ToxA, O-T-G-DIbK, O-T-G-DIIIK, or O-T-G-PE38K to detect periplasmic or cellular lysis is shown in Figure 11. The phosphatase assay on supernatants containing the expressed WT ToxA or O-T-G-DIbK resulted in a decrease in phosphatase activity relative to the empty vector control (set at 100%), indicating a low level of bacterial lysis due to their expression. Expression of O-T-G-DIIIK and O-T-G-PE38K led to a 3-fold increase in phosphatase activity relative to the empty vector demonstrating a substantial amount of lysis due to their expression. The results of the phosphatase assay are congruent with the amount of pelleted protein observed through PRMM precipitation of supernatant, with O-T-G-DIIIK and O-T-G-PE38K having considerably large pellets relative to the expression of WT ToxA and O-T-G-DIbK, including its counterpart O-T-G-DIbR (REDLK ER retention signal) (data not shown).
Figure 11. Phosphatase assay on bacterial supernatants expressing WT ToxA and TGFα:ToxA chimeras. Supernatants of arabinose induced VNP20009 harboring plasmids encoding WT ToxA, O-T-G-DIbK, O-T-G-DIIIK, and O-T-GPE38K were assessed for the presence of phosphatase activity. Raw data were normalized to empty vector plasmid control representing 100% activity (n = 2 for ToxA, O-T-G-DIbK, and O-T-G-DIIIK, n = 3 for O-T-GPE38K).
DISCUSSION

Summary and significance of results

_Salmonella_ strain VNP20009 has an innate ability to colonize and suppress tumor growth in mice. However, the outcome of a human clinical trial that utilized the VNP20009 strain on individuals affected by melanoma did not demonstrate the ability of the bacteria to cause tumor regression, though bacteria were present in the tumors (Toso et al., 2002). The reason for the lack of antitumor effect in humans is unknown, but it is possible that the level of attenuation VNP20009 contains may weaken the strain to the extent that it cannot overcome the human immune system or perhaps a different tumor type may have been a more suitable target.

_In vitro_ studies utilizing VNP20009 have shown that the bacteria induce apoptosis in tumor cells (Ganai et al., 2011) and the results of the work presented in this thesis demonstrate that its culture supernatant has an insignificant effect on cancer survivability, but can be enhanced through expression of a targeted toxin. As described by Kasinkas and Forbes (2006), tumor cylindroids, which are proposed to mimic the tumor environment _in vitro_, were utilized to demonstrate that _Salmonella_ chemotactically targets and accumulates within cylindroids containing cancer cells. However, internal accumulation only occurred in large cylindroids (784 μM diameter) comprised of heterogenous (proliferative, quiescent, and necrotic) cells. Only peripheral targeting was observed when small cylindroids (256 μM) comprised of proliferative cells alone were used, which presumably lacked or had a low concentration of internal chemo-attractants.
such as ribose and/or galactose that accumulate through nuclear degradation of necrotic cells (Kasinkas and Forbes, 2007). The inability of the bacteria to penetrate the cylindroid in this case is an interesting result that may explain the lack of success VNP20009 had in the clinical trial. Delivery of a greater number of bacteria may improve efficacy through increased physical interaction between the bacteria and tumor cells, with the caveat of increasing the likelihood of bacterial induced sepsis. Enhancing VNP20009 by engineering it to express a chimeric targeted toxin was hypothesized to be a safer approach and could compensate for the inability of the bacteria to cause tumor regression in humans by producing the therapeutic protein in a continuous and sustained manner (Quintero et al, 2016). Should the bacteria localize only to the periphery of the tumor, the targeted toxin may readily diffuse to areas where the bacteria may not approximate themselves close enough to induce apoptosis on their own.

Many of the studies on TGFα:ToxA chimeras utilized protein harvested from bacterial inclusion bodies that were unfolded then refolded chemically (Siegall et al., 1989a). In a number of those studies it was demonstrated that refolded protein taken from inclusion bodies were approximately 10-fold more potent than naturally folded protein taken from the periplasm of E. coli (Siegall et al., 1989a). In order to establish a live tumor-targeting Salmonella/targeted-toxin delivery system, synthesis of active therapeutic proteins must be wholly processed and secreted or released by the bacteria themselves. Therefore, TGFα:ToxA chimeric sequences taken from the literature, with the prerequisite of having both solubility and toxicity when harvested from the periplasmic space, were further modified to enhance their release into the media by Salmonella str. VNP20009.
In this work, cytotoxicity was assessed through the direct use of bacterial supernatants, harvested from broth cultures of *Salmonella* expressing toxins, to closer represent the activity of the secreted compound with minimal disturbance or alteration of the protein structure. Initial assays using the bacteriological media LB resulted in toxicity towards the MDA-MB-468 cell line. Because this bacterial media induced toxicity could mask any toxicity associated with the actual expression of a secreted or released toxin, the constituents of LB (1% tryptone, 0.5% yeast extract, 1% NaCl) were tested individually against the cancer cell line MDA-MB-468. TSB, MH broth, and M56 minimal media + 0.4% casamino acids and its components, were also assessed to determine their potential as alternative bacteriological media. As shown in Figure 2, 1% NaCl and 0.4% casamino acids did not contribute any toxicity towards the MDA-MB-468 cells and thus were considered for a new bacterial growth media preparation. Due to initial screening using *Salmonella* str. JR501, which is auxotrophic for tryptophan production (*trpC*), 0.2% tryptone was incorporated with 0.2% casamino acids due to the lack of tryptophan in casamino acids through destruction of the amino acid in the hydrolysate preparation (Mueller and Johnson, 1941). Additionally, 0.1% yeast extract was included to provide vitamins and nutrients, specifically the purine adenine to allow for growth of the *purI* mutant, VNP20009, and 0.9% NaCl to provide isotonicity. This novel bacteria media, named CTY (Casamino acids, Tryptone, Yeast), was shown to be conducive to bacterial protein expression, while showing remarkably low toxicity towards MDA-MB-468, as demonstrated through comparison with PBS. Furthermore, the results indicate that any proteins naturally secreted by VNP20009 had an insubstantial effect on the cancer cells, and any inhibitory or proliferative effect produced by the
supernatant would be solely due to the inducible expression of a secreted or released effector protein. The development of CTY was fundamental for continued experimentation using bacterial supernatants.

Initial testing included induced culture supernatants of VNP20009 harboring a WT ToxA expression vector that confirmed its ability to express and secrete an active form of toxin, which was reported in previous study (Swofford et al., 2014). The work in this thesis extends that study to include expression of TGF\(\alpha\):ToxA genetic fusions (Edwards et al., 1989; Kihara and Pastan, 1994; Kreitman et al., 1992) that were further modified for bacterial secretion or release. Each TGF\(\alpha\):ToxA chimera may have varying ToxA specific epitopes due to different truncations made to the WT toxin for each individual fusion. Therefore, quantification of the chimeras was done through monoclonal antibody detection of TGF\(\alpha\) (instead of the WT ToxA polyclonal), which is shared amongst all the chimeric constructs, to determine the protein concentration and cytotoxic activity contained within the culture supernatant. Based on those assessments, the culture supernatant of VNP20009 expressing O-T-G-PE38R was the most potent, producing the least amount of protein with the highest activity (Figures 4 and 5). The expression of the O-T-G-DIbR construct did not achieve the degree of cell killing that O-T-G-PE38R did through delivery of active supernatant (Figure 4), yet produced a much greater amount of protein (Figure 5). Culture supernatant from the expression of O-T-G-DIIIIR sequence did not lead to either significant toxicity or protein production, but was more potent than O-T-G-DIbR based on immunoblot quantification.

Due to the greater potency of O-T-G-PE38R, it was chosen for further analysis through modulation of its active peptide sequences and domains. Substitution of the WT
C-terminal endoplasmic reticulum retention signal, REDLK, for its mammalian counterpart, KDEL, is a modification that has been extensively studied and shown to enhance toxicity (Kihara and Pastan, 1994b). Comparably, the O-T-GPE38K chimera also had enhanced cytotoxicity when expressed in VNP20009 (Figure 6). An N-terminal deletion analysis was conducted on the O-T-GPE38K construct to assess the activity of each of its components and its contribution towards toxicity. The OmpA signal sequence, which is necessary for secretion and the TGFα ligand which binds EGFR, were required for toxicity as previously reported (Kreitman et al., 1992) (Figure 7). Expression of the OmpA deletion (ΔOmpA) construct was undetected by western blot, while the TGFα deletion (ΔTGFα) construct was detected at a lower molecular weight relative to O-T-GPE38K, due to the TGFα truncation, confirming that the signal peptide is necessary for secretion and that TGFα is required for toxicity.

A subsequent deletion to the long flexible linker introduced was utilized to determine its effect on the toxicity of the chimera. A previous study demonstrated a 700-fold increase in activity in a TGFα:DIIIK fusion when the short flexible linker GGGGS was added and sample taken from the periplasm of E. coli (Kihara and Pastan, 1994). A ToxA chimera composed of PE38K and an affibody targeting the Her2 receptor also benefited from the flexible linker GGGGSGS (Zielinski et al., 2009). Additionally, an EGFR targeting peptide bound to polyethylenimine (PEI) was separated by a longer (GGGS)₃ linker, which led to enhanced cell permeability (Li et al., 2005). The culture supernatant from VNP20009 expressing the chimera with a deletion of the long flexible linker (Δ3GS) resulted in a loss of activity demonstrating that its presence indeed enhances activity (Figure 7). Accumulation of the linker-less chimera was detected in the
supernatant through western blot, but at a much lower level than the complete construct O-T-G-PE38K. The increase of protein in the media detected through expression of unmodified O-T-G-PE38K construct may be due to presence of the long flexible linker, which are known to provide enhanced folding and solubility, reducing proteolysis and increasing protein yield (Chen et al., 2013).

A single amino acid substitution of glutamic acid for aspartic acid (E553D) within the active site of the toxin, known to substantially reduce toxicity (Douglas and Collier, 1987), caused a complete loss of activity in WT ToxA (Figure 4). This finding demonstrates that even in the presence of an inactivated toxin the bacterial culture supernatant had no effect on tumor cells. Generally, TGFα is considered a mitogen that leads to proliferative effects. However, TGFα has been shown to induce apoptosis in MDA-MB-468 breast cancer cells at relatively high concentrations (Wang et al., 1997). To ensure that TGFα was not contributing to the toxicity observed, an inactivating mutation of O-T-G-PE38K through the E553D substitution within the active site of the toxin fragment was constructed (Figure 7). Expression of the O-T-G-PE38K-E533D construct resulted in a culture supernatant that completely lost its toxic activity, while the chimera was still detected through immunoblot, indicating that the toxicity of the unmodified O-T-G-PE38K is attributed to the C-terminal PE38K domain and not TGFα.

Co-expression of the pColE3 lysis protein was assessed based on studies that have suggested that Salmonella vector based delivery of therapeutic proteins could be enhanced by such genetic modifications (Bermudes et al., 2001A). The pColE3 lysis protein facilitates periplasmic secretion or release by activating outer-membrane porins and phospholipase activity causing the outer membrane to become increasingly
permeable (Carvard, 2004). Co-expression of the ColE3 lysis protein further enhanced the activity of O-T-G-PE38K greater than 3-fold and led to an increase in the amount of protein detected through western blot. Taken together, the modifications introduced into O-T-G-PE38K resulted in a supernatant that is more active than the supernatant containing the wild type toxin and may translate into a modular and potent targeted therapy.

Because O-T-G-PE38K supernatants were as toxic as WT ToxA, both toxin containing supernatants were delivered to cell lines expressing various amounts of EGFR to assess for specificity. These included MDA-MB-468 (high level EGFR), HeLa (low level EGFR), and H460 (no expression), whose expression level were confirmed by western blot. The results of the viability assays indicated a correlation of toxicity with the presence of EGFR expression for O-T-G-PE38K, but not for WT ToxA (Figure 8 and 9), which are consistent with the previous studies using TGFα:ToxA fusions (Siegall et al., 1989b).

The toxic effects of WT ToxA and ToxA chimeras are widely known to occur through the inhibition of the eukaryotic translational machinery through ADP-ribosylation of eEF2 (Weldon and Pastan, 2011). A less known mechanism is ToxA’s ability to trigger the apoptotic pathway through depletion of Mcl-1 that allows accumulation and activation of Bak, which causes increased mitochondrial permeability resulting in the activation of the caspase pathway inducing apoptosis (Du et al, 2011, Andersson et al., 2004 and Jenkins et al., 2004). Mitochondria are normally in a polarized state with a highly negative potential (> -240 mV) and are the most negatively charged organelles in mammalian cells (Michelakis, 2008). Mitochondrial membrane integrity
can therefore be monitored through the use of dyes specific for the negatively charged (polarized) mitochondria. JC-10 is a cationic dye with high selectivity for the negatively charged mitochondria, transitioning from a monomeric form in the cytosol to aggregate formation within the mitochondria (Patel et al, 2016). Depolarization events cause the mitochondrial transition pore to be activated allowing pro-apoptotic mediators to enter the cytosol causing induction of the apoptotic pathway in mammalian cells as well as changing the mitochondrial potential to a more positive state (Michelakis, 2008). Depolarized mitochondria are therefore indicative of cells undergoing apoptosis and thus the mitochondrial stain JC-10 was utilized to quantitate depolarization events in cells treated with supernatants from VNP20009 harboring an empty vector plasmid, and WT ToxA or O-T-G-PE38K constructs.

The low-EGFR expressing cell line, HeLa, showed a reduction in the JC-10 aggregate signal only when treated with WT ToxA, which is consistent with the results of the MTT cell viability assays (Figure 10). Though O-T-G-PE38K did not cause a depolarization event in the HeLa cells, it did cause an opposite, hyperpolarization event, which has been characterized as a mechanism of circumventing the induction of apoptosis (Beltran et al., 2002). Understanding this phenomena is worth future exploration because it may or may not lead to the discovery of anti-apoptotic pathway, which can be co-targeted along with O-T-G-PE38K to enhance toxicity in non-responsive EGFR expressing cell lines. The high-EGFR expression cell-line MDA-MB-468 showed a depolarization state in both the ToxA and O-T-G-PE38K treatment groups. O-T-G-PE38K led to a significantly larger reduction in the mitochondrial signal than ToxA, due to its apparent higher cytotoxic potency against the MDA-MB-468 cell line.
In order to further assess the potential activity of the other constructs, O-T-G-DlbR and O-T-G-DIIIR, the C-terminal REDLK ER retention signal was replaced with the mammalian KDEL consensus sequence and also co-expressed with the pColE3 lysis protein. Activity increased for both constructs harboring the two genetic modifications with the O-T-G-DlbR construct benefiting the most. O-T-G-DlbK (KDEL) gained an increase in activity over O-T-G-DlbR and marked increase when co-expressed with the CoLE3 lysis protein. Supernatant activity from the expression of the O-T-G-DIIIR also benefitted from the same modifications but did not achieve the levels of toxicity seen with O-T-G-DlbR.

During the process of precipitating the total supernatant protein using the PRMM method, protein pellets of widely varying sizes were observed (data not shown). These observations were construct specific, with O-T-G-DIIIR(K) and O-T-G-PE38R(K) having markedly larger pellets than the induced supernatants from VNP20009 harboring an empty vector plasmid, WT ToxA, or O-T-G-DlbR(K). As shown in Figures 3 and 5, the amount of target protein for O-T-G-DIIIR and O-T-G-PE38R is markedly reduced relative to O-T-G-DlbR. Therefore, the differences in the accumulation of precipitated protein was suspected to have been due to bacterial lysis and not to overexpression of the target protein.

A phosphatase assay (Brickman and Beckwith, 1975) was utilized to determine the relative amount of bacterial lysis each construct caused due to their expression. As indicated in Figure 11, the expression of WT ToxA or O-T-G-DlbK caused less accumulation of the phosphatase(s) enzyme activity in the supernatant relative to an empty vector plasmid control. This result may be due to a decrease in bacterial growth.
kinetics of the bacteria harboring these vectors, which have the burden of expressing WT ToxA and O-T-G-DIbK, contributing to less leakage of cellular proteins through less frequent cell division. On the contrary, O-T-G-DIIIK and O-T-G-PE38K significantly increased the phosphatase activity within the bacterial supernatant, which is in line with the observations made when precipitating total protein. O-T-G-DIbK contains nearly the entire domain Ib (DIb) protein sequence of the WT ToxA except for the four N-terminal amino acids. This sequence includes two cysteines that form a disulfide bond in WT ToxA. To date, there is no actual known activity for DIb other than possessing four C-terminal amino acids necessary for full ADP-ribosylation activity (Siegall et al, 1989).

DIb has been proposed to contribute to internalization of the WT ToxA through its close approximation with domain Ia (DIa) that binds the α-2 macroglobulin receptor. It has also been proposed that DIb contributes to the secretion of WT ToxA, though this has not been explored (Siegall et al, 1989). Interestingly O-T-G-DIbR(K) is nearly identical to O-T-G-DIIIR(K) except the latter lacks the majority DIb protein sequence including both its cysteine residues; a difference of 16 amino acids. O-T-G-PE38R(K) contains a similar deletion within the sequence encoded by DIb that also lacks the cysteine residues. This small section of protein sequence in O-T-G-DIbR(K) is enough to prevent the bacterial lysis observed in both O-T-G-DIIIR(K) and O-T-G-PE38R(K), at levels observed with the non-lytic expression of WT ToxA that contains the intact DIb sequence. Expression of O-T-G-DIbR was shown to have accumulated the most protein among the chimeric constructs reported using either an anti-ToxA or anti-TGFα antibody (Figures 3 and 5). These findings lend support to the proposed idea that DIb may play a role in the secretion process of WT ToxA and seems to provide a major contribution to
the secretion of VNP2009 expressed O-T-G-DIbR(K). In terms of a bacterial delivery system, expression of therapeutic compounds should not hinder the bacteria’s ability to proliferate. The fact that O-T-G-DIbR(K) construct is expressed without causing bacterial lysis and leads to a higher concentration of the protein in the supernatant merits further exploration through modifications that may enhance its toxic activity.

**Future Directions**

Future studies could entail the use of the expression plasmids created in this work to explore new combinations of signal peptides, receptor ligands, linkers and toxin domains. These plasmid vectors were designed to be modular with restriction sites made available to easily substitute new sequences (Table 2) that may enhance activity or used to target different receptors for novel drug design.

Signal sequences other than the Sec dependent OmpA signal utilized in this thesis may or may not enhance secretion of ToxA chimeras. Alternative secretion signals may be derived from MalE, PelB, and PhoA, (Sec dependent pathway), DsbA, TorT, and TolB, (SRP pathway) and Pac or TorA (TAT pathway) that have been shown to deliver proteins of interest within the periplasm or extracellular milieu in active forms (Yoon et al., 2010). Fusions to carrier proteins offer alternative pathways to secretion of an active ToxA chimera and may include fusions to YebF, MalE, OmpF, and HlyA (Yoon et al., 2010). Though there is no guarantee that the use of these alternative signal sequences or fusions to carrier proteins will lead to an increase in toxicity of ToxA chimeras already demonstrated through this work, the possibility does exist and the tools are available to construct theses alternative forms utilizing the pre-established vectors.
The EGFR binding ligand, epidermal growth factor (EGF), has been utilized as an alternative EGFR binding ligand and has previously been fused to truncated ToxA sequences as an N-terminal fusion (Liao et al., 1995) and a C-terminal fusion to PE40, which had the EGF peptide sequence modified at key residues to enhance binding activity (Shiah et al., 1992). Those constructs were purified from whole cell lysates of *E. coli* and chemically folded to achieve activity. However, active recombinant EGF has been harvested from the periplasmic space when fused to an N-terminal OmpA signal sequence (Abdull et al., 2008) and excreted into the bacterial supernatant in an active form when fused to the *Yersenia pestis* Caf-1 signal sequence (Liu et al., 2006), which may indicate that toxic activity within the bacterial supernatant may be achieved if incorporated into one of the constructs described in this thesis. Additionally, EGF mutants with faster binding kinetics have been created (Lahti et al., 2011), and fusion of those peptide mutants to a truncated ToxA may enhance toxicity towards EGFR expressing cell lines.

Alternatively, an EGFR binding peptide (EBP), sequence YHWYGTPQNV, acquired through screening of M13 phage display libraries (Li et al., 2005), binds EGFR with good kinetics ($K_d \sim 22$ nM) but does not have the mitogenic effects associated with TGFα or EGF. It has the additional benefit of not containing cysteines that form a disulfide bond, which may or may not enhance the solubility and secretion of ToxA chimeras. An N-terminal fusion of the EBP to PE38K has already been constructed as a peripheral part of this project using the M13 pIII and OmpA secretion signals. Evaluation of its toxicity towards EGFR-overexpressing cell lines has not been completed and the other ToxA truncations remain be fused to the EBP. C-terminal fusions of EBP to a
truncated ToxA may also be constructed if activity is not present with N-terminal variants, though these fusions would require the REDL(K) or KDEL at the end of chimeric sequences for retrograde trafficking. In addition to EGFR targeting, Zielinski et al., 2009 generated a ToxA chimera that targets the Her-2 receptor (a member of the EGFR receptor kinase family) that has demonstrated specific activity against Her-2 overexpressing cell lines. There are several other ligands available that can be fused to ToxA to produce a targeted toxin, which may provide enhanced activities against cell lines over-expressing the cognate receptor.

As previously discussed, flexible linkers composed of glycine and serine residues have been broadly explored in ToxA chimeras and shown to improve activity (Kihara and Pastan, 1994, Zielinski et al., 2009, and Quintero et al., 2016). Adjusting the length of linkers have improved the activity of other specific fusions (Chen et al, 2013), which may also increase the activity of ToxA chimeras and may also be explored. Alternatively, the alpha helical linker (EAAAK)n and the rigid linker A(EAAAK)nA have also been employed to provide physical separation between domains (Chen et al, 2013). Cleavable linkers, which contain the proteolytic site of specific proteases, have been utilized to release the protein of interest from a fusion product in-vivo (Chen et al, 2010). Addition of the furin cleavage site (-RG/[Q]PR-) (Inocencio et al., 1994 and [Genbank WP_019485543]), that is embedded within domain II of WT ToxA, was shown to improve toxicity of ToxA chimeras that lack it (Weldon and Paston, 2011). Therefore, the furin site has been incorporated between the flexible linker (3GS) and domain Ib (DIb) of O-T-G-DIbK as a means to improve toxicity and may be evaluated for its toxicity against EGFR over-expressing cell lines. The furin site may also be moved central to the flexible
linker or at its N-terminus or positioned in combination with other linkers and chimeric sequences to assess for improved activity.

Similarly, proteolytic activation of the ToxA chimeras can be achieved by replacing the furin cleavage site for a protease site specific to proteases overexpressed by tumor cells (Pastan et al., 2007). Additionally, the ToxA sequence may be modified such that lysines, which are ubiquitinated for proteasome degradation, are no longer available and may improve stability of the ToxA chimera (Weldon and Pastan, 2011). Assessment of ToxA chimeras in this thesis may be further extended through use of other truncated ToxA fragments in the literature (Siegal et al., 1989). Additionally, TGFα or other ligand fusions to toxins such as diphtheria toxin, ricin, granzyme B or ribonucleases (Pastan et al., 2007) may also be considered as targeted therapies that may be secreted or released by Salmonella.

Co-expression of the disulfide bond isomerases DsbA and DsbC and their modulators DsbB and DsbD, respectively, are known to improve folding efficiencies of proteins directed into the periplasm. In particular the human nerve growth factor (hNGF), which has a convoluted folding pattern highly similar to TGFα and EGF, was shown to accumulate in periplasm in an active form when fused to an OmpT signal and co-expressed with DsbCD and further accumulated with co-expression of DsbABCD (Kurokawa et al., 2001). Therefore, co-expression of disulfide bond isomerase may be utilized as a means to enhance the toxicity of the TGFα:ToxA chimeras or others.

Novel bacterial secreted therapeutics such protease inhibitors (PIs) may inhibitor tumor-associated proteases and/or pro-inflammatory processes. The sunflower trypsin inhibitor-1 (SFTI-1) derived from the sunflower plant is a small 14 amino-acid bicyclic
peptide that potently inhibits the serine protease trypsin and the tumor associated trypsin-like protease matriptase, which is correlated with aggressive metastatic tumors of the breast, cervix, ovaries, prostate and liver (Cai et al., 2011). Matriptase over-expression or its under-modulation by its cognate inhibitor hepatocyte activator inhibitor-1 (HAI-1) (Lebeau et al., 2012) leads to activation of several proenzymes involved in tumor cell proliferation such as the urokinase-type plasminogen activator (uPA), which is known to cause tissue remodeling, tumor cell invasiveness and angiogenesis in cancer, and hepatocyte growth factor (HGF) a mitogen that leads to a motile phenotype, morphogenic changes and also induces tumor angiogenesis (Uhland, 2006). Additionally, matriptase activates the protease activated receptor-2 (PAR-2), a G-protein coupled receptor involved in cellular adhesion and inflammatory processes (Takeuchi et al., 2000).

Because of the major role matriptase plays in proliferative tumor and pro-inflammatory effects, a C-terminal genetic fusion of SFTI-1 with the secreted carrier protein YebF (Zhang et al., 2006) was constructed as a potential inhibitor of matriptase and other trypsin-like proteases that may be delivered by VNP20009 in situ. Furthermore, a secreted version of YebF-SFTI may inhibit PAR-2 activation as shown with other serine protease inhibitors, which decreased the motility of highly metastatic cell line MDA-MB-231 breast cancer that secretes a trypsin-like protease by inhibiting its proteolytic activity (Ge et al., 2004). Secretion of the protease inhibitor elafin by Lactococcus and Lactobacillus strains was shown to reduce the inflammation in guts of mice that was induced by dextrose sodium sulfate (DSS) and have been engineered as potential therapeutic bacteria (Motta et al., 2012). Hypothetically, a functionally secreted YebF-SFTI chimera may also be utilized as an anti-inflammatory therapy for the
treatment of inflammatory bowel disease (IBD) through inhibition of PAR-2 known to be activated through trypsin-like protease, which may be delivered by a probiotic such as the *E. coli* Nissle str. 1917 that has been engineered to deliver other therapeutics in the gut (Chen et al., 2014). These YebF-SFTI chimeras have shown strong inhibitory activity utilizing a culture based assay for the detection of bacterial secreted protease inhibitors (Quintero and Bermudes, 2014) and *in vitro* using bacterial supernatants and the chromogenic trypsin substrate Nα-Benzoyl-D,L-arginine p-nitroanilide hydrochloride (BApNA) (Sigma Aldrich) (Quintero and Bermudes, 2016). Purification of YebF-SFTI is in progress. Using the ammonium sulfate (AS) precipitation method, YebF-SFTI was found in the 50% - 80% AS fraction and will be desalted with a Sephadex G-10 column, then further purified by other means if necessary and specific activity quantified. Hexa-His tag versions of the YebF-SFTI chimera have also been constructed as an alternative means of purification through column purification or analysis of the stability of the construct through western blot.

SFTI-1 sequences have been permutated to achieve increased inhibitory activity against matriptase and variants have been produced with a 64-fold to a 350-fold increase over wild type (Fittler et al, 2014). This finding is particularly important as the mutants were without the bicyclic structure characteristic of SFTI-1, though they do contain the single disulfide bond (mono-cyclic), which may represent the true structure of the YebF-SFTI chimera. These SFTI-1 mutants may improve the inhibitory activity already demonstrated with YebF-SFTI and may be constructed in future studies.

Expression of YebF-SFTI by VNP20009 showed strong inhibitory activity against trypsin and when co-expressed with O-T-G-PE38K, demonstrated activity from
both proteins in vitro (Quintero and Bermudes, 2016). A polycistronic sequence containing O-T-G-PE38K, YebF-SFTI, and the ColE3 lysis was also constructed and showed activity from each component in the genetic construct. Active chimeras such as O-T-G-DIbR(K) O-T-G-DIIIR(K) and O-T-G-PE38R(K) may be inserted into the chromosome of VNP20009 to assess for their activity as a single copy, through the use of a sucrase vector or the Lambda Red Recombinase system (Datsenko and Wanner, 2000) similar to what was used to generate the derivative of VNP20009, TAPET-CD (King et al., 2009). These insertions may also include the sequences of the functional secreted protease inhibitor YebF-SFTI and pColE3 lysis protein to generate an inducible operon for the selective destruction of tumor cells by tumor-targeted Salmonella.
REFERENCES


Cavard, D. Role of Cal, the colicin A lysis protein, in two steps of colicin A release and in the interaction with colicin A–porin complexes. Microbiology 2004. 150: 3867-3875.


ClinicalTrials.gov Identifier: NCT00004988, Treatment of patients with cancer with genetically modified Salmonella typhimurium bacteria.

ClinicalTrials.gov Identifier: NCT01099631, IL-2 expressing, attenuated Salmonella typhimurium in unresectable hepatic spread.

ClinicalTrials.gov Identifier: NCT01118819, Safety study of Clostridium novyi-NT spores to treat patients with solid tumors that have not responded to standard therapies.

ClinicalTrials.gov Identifier: NCT01598792, Safety study of recombinant Listeria monocytogenes (Lm) based vaccine virus vaccine to treat oropharyngeal Cancer.


Du, X., Youle, R.J., Fitzgeral, D.J., Pastan, I. Pseudomonas exotoxin A-mediated apoptosis is Bak dependent and preceded by the degradation of Mcl-1. Molecular and Cellular Biology 2010. 30 (14): 3444-3452

Edwards, G.M., DeFeo-Jones, D., Tai, J.Y., Vuocolo, G.A., Patrick, D.R., Heimbrook, D.C., Oliff, A. Epidermal growth factor receptor binding is affected by structural determinants in the toxin domain of transforming growth factor-alpha


Hessler, J.L., and Kreitman, R.J. An early step in *Pseudomonas* exotoxin action is removal of the terminal lysine residue, which allows binding to the KDEL-receptor. Biochemistry 1997. 36:14577-14582


Kreitman, R.J., and Pastan, I. Importance of the glutamate residue of KDEL in increasing the cytotoxicity of *Pseudomonas* exotoxin derivatives and for increased binding to the KDEL receptor. Biochemical Journal 1995. 307: 29-37


Kurokawa, Y., Yanagi, H., and Yura, T. Overproduction of bacterial protein disulfide isomerase (DsbC) and its modulator (DsbD) markedly enhances periplasmic


Lorberboum-Galski, H., Kozak, R.W., Waldman, T.A., Bailon, P., Fitzgerald, D.J.P.,
Pastan, I. Interleukin 2 (IL-2) PE40 is cytotoxic to cells displaying either p55 or
p70 subunit of the IL-2 receptor. Journal of Biological Chemistry 1988. 263(35):
18650-18656

Lorey, S., Strom, M.S., and Johnson, K. Expression and secretion of the cloned

\textit{Pseudomonas aeruginosa} exotoxin A by \textit{Escherichia coli}. Journal of Bacteriology

Low, K.B., Ittensohn, M., Le, T., Platt, J., Sodi, S., Amoss, M., Ash, O., Carmichael, E.,
Chakraborty, A., Fischer, J., Lin, S.L., Luo, X., Miller, S.I., Zheng, L-m., King, I.,
Pawelek, J.M., Bermudes, D. Lipid A mutant \textit{Salmonella} with suppressed
virulence and TNF\alpha induction retain tumor-targeting in vivo. Nature
Biotechnology 1999. 17 (1): 37-41

Luo, X., Li, Z., Lin, S., Le, T., Ittensohn, M., Bermudes, D., Runyab, J.D., Shen, S.Y.,
Chen, J., King, I.C., Zheng, L.M. Antitumor effect of VNP20009, an attenuated
\textit{Salmonella}, in murine tumor models. Oncology Research Featuring Preclinical
and Clinical Cancer Therapeutics 2001. 12(11-12): 501-508

McInnes, C., Wang, J., Al Moustafa, A-E., Yansouni, C., O’Connor-McCourt, M., Sykes,
B.D. Structure-based minimization of transforming growth factor-\alpha (TGF-\alpha)
through NMR analysis of the receptor-bound ligand. Journal of Biological


Morales, M., Attai, H., Troy, K., Bermudes, D. Accumulation of single-stranded DNA in Escherichia coli carrying the colicin plasmid pColE3-CA38. Plasmid 2014. 77: 7-16


Quintero, D., and Bermudes, D. A Chimeric protease inhibitor secreted by *Escherichia coli* with the potential for use in biofuel fermentations and therapeutic bacterial delivery vectors. Poster presented at: ASM Microbe 2016; 2016 Jun 16-20; Boston, MA, USA.


Quintero, D., Carrafa, J. Vincent, L., Bermudes, C. EGFR-targeted chimeras of *Pseudomonas* ToxA released into the extracellular milieu by attenuated *Salmonella* selectively kill tumor cells. Biotechnology and Bioengineering 2016. DOI: 10.1002/bit.26026


APPENDIX

Computer program for fusion of TGFα:PE40 Python language

# Classes

class Atom:  
'a unified atom or hetatm class for pdb data'  
# format information available here  
http://deposit.rcsb.org/adit/docs/pdb_atom_format.html  
def __init__(self, atm): # build from an input line  
'parse an ATOM or HETATOM line'  
self.atomSerial = int(atm[6:11])  # fields in the ATOM record  
self.atomName = atm[12:16].lstrip(' ').rstrip(' ')  
self.altLoc = atm[16] # Alternate location indicator  
self.resiName = atm[17:20].lstrip(' ').rstrip(' ')  
self.chainID = atm[21]  
self.resiSeq = int(atm[22:26]) # Residue sequence number  
self.resiInsert = atm[26] # Code for insertion of residues  
self.x = float(atm[30:38]) # Orthogonal coordinates for X in Angstroms  
self.y = float(atm[38:46]) # Orthogonal coordinates for Y in Angstroms  
self.z = float(atm[46:54]) # Orthogonal coordinates for Z in Angstroms  
self.occupy = float(atm[54:60]) # Occupancy  
self.temp = float(atm[60:66]) # Temperature factor (Default = 0.0)  
self.segID = atm[72:76] # Segment identifier, left-justified  
self.eltSymb = atm[76:78].lstrip(' ').rstrip(' ') # Element symbol  
self.charge = atm[78:80] # Charge on the atom

class Residue:  
'a collection of atoms - could be an amino acid or sugar or even water'  
torsion = list() # placeholder for sdtorsion angles  
def __init__(self, atmList, longResidueNames): # build from a list of atomSerials  
self.list = atmList  
self.resiName = self.list[0].resiName # use the first atom on the list as an example, to record name and sequence of residue  
self.resiSeq = self.list[0].resiSeq  
self.resiInsert = self.list[0].resiInsert
try:
    self.longName = longResidueNames[self.resiName]
except:
    pass  # not all residues may have recorded "long names",
because they're gathered from HETNAM
self.name2atom = dict()  # build a dict of atomName (keys)
pointing to atom object (value)
    for a in atmList:
        self.name2atom[a.atomName] = a

    # so that you can use it in a for loop
    def __len__(self):
        'allow the len function to return the number of atoms in the
        residue'
        return len(self.list)  # return number of atoms

    def getAtom(self, atomName):
        'take the atom name and return the atom object'
        for thisAtom in self.list:
            if atomName == thisAtom.atomName:
                return thisAtom
        return 0  # didn't find

    # prints all atom names within the residue (used for testing)
    def printNames(self):
        for a in self.list:
            print(a.atomName)

    # return the Atom object at a specific index. Parameter =
    integer
    def getAtomAtNdx(self, ndx):
        return self.list[ndx]

##########################################################################
###   MAIN PROGRAM
##########################################################################

# get all lines from the 1IKQ
lines = []
f = open("1IKQ.pdb", 'r')
lines.extend(f.readlines())
f.close

# get all atoms
atoms = []
for k in range(537, 5173):  # for each atom line
    line = lines[k];
    atoms.append(Atom(line))

# create residues from atoms
residues = []
done = 1
count = 0
rs = atoms[count].resiSeq # current residue sequence number in Atoms list
atomsInResidue = [];
atomsInResidue.append(atoms[0])
count = count + 1
while done == 1:
    # check if a new residue sequence is encountered
    if atoms[count].resiSeq != rs:
        residues.append(Residue(atomsInResidue,
            atomsInResidue[0].resiName))
        atomsInResidue = [] # clear the list
        rs = atoms[count].resiSeq
        atomsInResidue.append(atoms[count])
        count = count + 1
        if count >= (len(atoms) - 1):
            done = 0

# get residues from 1YUF file
lines = []
f = open("1YUF.pdb", 'r')
lines.extend(f.readlines())
f.close

# get all atoms
atoms = []
for k in range(480, 1199): # from model 1
    line = lines[k];
    atoms.append(Atom(line))

# shift all atoms for binding to IKQ
for a in atoms:
    a.x = round(a.x + 17.5337, 3) # round(number, decimal places)
    a.y = round(a.y + 63.17, 3)
    a.z = round(a.z + 3.047, 3)

# create residues from atoms for YUF
YUFresidues = []
done = 1
count = 0
rs = atoms[count].resiSeq
atomsInResidue = [];
atomsInResidue.append(atoms[0])
count = count + 1
while done == 1:
    # check if a new residue sequence is encountered
    if atoms[count].resiSeq != rs:
        YUFresidues.append(Residue(atomsInResidue,
            atomsInResidue[0].resiName))
        atomsInResidue = []
        rs = atoms[count].resiSeq
        atomsInResidue.append(atoms[count])
        count = count + 1
        if count >= (len(atoms) - 1):
            done = 0
# truncates the residues (domain 1) from the front of 1IKQ
residues = residues[244:] # [starting:ending]

# put residues of 1YUF in front of 1IKQ
residues.extend(YUFresidues)

# write residues into a new file
f = open("new.pdb", 'w')
count = 1
for r in residues:
    for atom in range(0, len(r)):
        a = r.getAtomAtNdx(atom)
        line = "{:<6}{:>5}{:^6}{:<4}{:1}{:>4}{:>5}{:>7}{:>8}{:>8}{:>6}{:>6}{:>10}{:>
2}".format("ATOM",str(count),str(a.atomName),str(a.resiName),str(a.
chainID),str(a.resiSeg),str(a.resiInsert),str(a.x),str(a.y),str(a.z)
,str(a.occupy),str(a.temp),str(a.segID),str(a.eltSymb),str(a.charge)
)
        f.write(line)
        count = count + 1
        f.write("\n")
f.close()