Journal of Analytical & Bioanalytical Techniques

Richa, J Anal Bioanal Tech 2023, 14:3

Mini Review Open Access

# Targeted Protein Delivery Using Modified Anthrax Toxin Protective Antigen

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## Abstract

**Background:** It would be highly desired to develop effective strategies for trans locating tailored binding proteins to the cytosol since they might target flat and hydrophobic protein-protein interfaces, which would expand the amount of the druggable proteome. Two levels of specificity would be gained, one for the cell type and the other for the cytosolic target, if this could be done in a way that was dependent on a cell surface receptor. Such mechanisms have naturally developed in bacterial protein poisons. Protective antigen (PA), a translocation unit that forms pores, and a distinct protein payload make up anthrax toxin. By combining a chosen ankyrin repeat protein with PA, it is possible to engineer PA to abolish binding to its own receptor and instead bind to a desired receptor, leading to uptake in different cell types.

**Results:** The amount of redirected PA that can be administered and subsequently the amount of delivered payload are both constrained by prepore-to-pore conversion of redirected PA that already takes place at the cell surface. We proposed that the deficiency of a stabilising interaction with the wild-type PA receptor is the cause. Now, PA has been redesigned to include the CMG2 anthrax receptor binding domain followed by a DARPin that binds to the desired receptor. This construct may be supplied at considerably higher concentrations without causing toxicity, undergoes prepore-to-pore conversion only in late endosomes, and is thus stable. As a result, it delivers substantially larger payload levels to the cytosol.

**Conclusion:** We think that this reengineered system represents a significant advancement in the quest for effective protein delivery to the cytosol in specific cells.

## Introduction

Today, targeted therapy is used to treat a variety of disorders, particularly those in which aberrant signalling is significant. Usually, there are two different kinds of molecular targets. The first category consists of antibodies that target cell surface molecules. These molecules have a range of methods of action, such as the suppression of signalling, the mobilisation of immune responses or other molecules, or they can be combined with toxins to create antibody-drug conjugates. The second class of pharmacological targets is intracellular, with kinases serving as an example. These tiny compounds, which are naturally cell permeable, target kinases and attach to specific locations on the target protein. Despite the fact that each of these strategies has showed significant promise, common issues include the insufficient therapeutic window and the quick development of resistance. Intracellular protein-protein interactions constitute a largely unexplored pool [1-6] of targets for cell-specific targeted therapy, in contrast to extracellular targets that are easily accessible to antibodies or other binding proteins. As a result of decades of progress in medicinal chemistry, small compounds can be created that have great selectivity and affinity for several intracellular proteins that provide pockets. Small molecules are unable to attach with sufficient specificity to hydrophobic and flat protein-protein interfaces that lack deep binding pockets, and as a result, they are typically unable to block protein-protein interactions.

## Materials and Methods

Small molecules may also be target-specific rather than cell-specific. While therapies are mainly restricted to targets accessible on the cell surface due to the plasma membrane's impermeability to biological macromolecules, including proteins, binding proteins can now be produced against practically any target molecule. With widely variable degrees of efficacy, numerous delivery techniques based on both naturally occurring and artificial systems are being developed to carry proteins across the plasma membrane, with the goal of expanding the druggable proteome. Bacterial protein poisons, such as the anthrax toxin (produced by Bacillus anthracis), have naturally evolved to get beyond this barrier, the plasma membrane, and [7-9] can deliver protein toxins to the cytoplasm of cells in a receptor-specific way. The anthrax toxin complex is ingested by receptor-mediated endocytosis following receptor binding and proteolytic activation. Cargo molecules are either transported directly to the cytosol in the endosomes or to intraluminal vesicles, where they are finally returned to the cytoplasm through back fusion. These toxins have a modular structure that allows for the engineering of domains for different cell selectivity and translocated cargo, making it a flexible system for protein delivery. A collection of model binding proteins may now be delivered to the cytosol of cells using a generic delivery mechanism that our team recently designed based on the anthrax toxin. Ankyrin repeat protein that is engineered to interact to the epithelial cell is used to retarget the cell-binding and translocation domain of the anthrax toxin. The PA-binding domain of one of the two natural anthrax toxin payloads, lethal factor 1-254 (LFN), was fused N-terminally to distinct cargo DARPins. Utilizing this retargeting method, we successfully delivered these cargo DARPins to the cytoplasm of EpCAM-expressing cells. For retargeted PA, however, only modest amounts could be employed, due to a cytotoxic impact of PA alone that occurred with higher concentrations (> 20 nM). Our aim

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**Received:** 01-Mar-2023, Manuscript No: jabt-23-90528, **Editor assigned:** 03-Mar-2023, Pre QC No: jabt-23-90528(PQ), **Reviewed:** 17-Mar-2023, QC No: jabt-23-90528, **Revised:** 21-Mar-2023, Manuscript No: jabt-23-90528(R), **Published:** 28-Mar-2023, DOI: 10.4172/2155-9872.1000505

**Citation:** Richa V (2023) Targeted Protein Delivery Using Modified Anthrax Toxin Protective Antigen. J Anal Bioanal Tech 14: 505.

was therefore to acquire an in-depth understanding of the underlying mechanism of this toxicity and use this information to design innovative reengineered protective antigen variants that overcome the cytotoxic constraints of retargeting and therefore to be able to deliver bigger quantities of payload. Motivated by the hypothesis that the interface between domains 2 and 4 in the wild-type PA prepore is fixed by binding to its natural receptor, we hypothesised that the cytotoxicity is most likely owing to a premature prepore-to-pore conversion of PA, already at physiological pH. To offset this impact, we now produced a stabilised variant of PA, which combines PA in its wild-type form with the wild-type soluble extracellular receptor-binding domain of PA, coupled to a retargeting DARPin. Here, we present a thorough protein characterisation, validate the eradication of the cytotoxicity, and show a greater absorption of cytosolically delivered proteins using the novel fusion design. We show that the amount of cytosolically delivered cargo was thus far limited by the cytotoxicity of the translocation domain and that this rate-limiting phase has now been overcome.

## Discussion

Because needs small binding sites needs small compounds small binding sites small molecules small molecules. The druggable prote proteome limit the drug drug prote prote prote prote prote prote prote prote. limit limit drug drug The absence of effective, cell-specific cytosolic delivery mechanisms currently prevents macromolecular binding molecules, which do not have this restriction, from being used. A solution to this issue would allow larger biological macromolecules, which may be produced to nearly any surface on the target, access to the intracellular target space. The availability of recombinant binding proteins today makes it possible for targeted therapeutic methods to become much more widespread. Then, as they lack a binding site for tiny compounds, many molecules of significant medicinal interest that are now thought to be "undruggable targets" could be addressed. This would make large, flat, and hydrophobic protein-protein interaction surfaces no longer impermeable.

## Results

Retargeting of PA to diverse cell surface receptors has previously been performed by fusing a binding protein to the C-terminus of PA, and we have developed such method employing DARPins. Having fused an EpCAM-targeting DARPin (Ac2) with an affinity of $1.3 \times 10^{-7}$ M to the C-terminus of a mutated version of PA, ablating binding to its own receptors, capillary morphogenesis gene-2 (CMG2) and tumour endothelial marker-8 (TEM8), we generated a highly efficient, cell-specific, retargeted delivery system. Even with low quantities (20 nM) of the retargeting fusion construct PAm-Ac2, we could identify the cytosolic presence of cargo DARPins. A structural bridge between domain 4 of PAwt and the von Willebrand factor A (VWA) region of the anthrax toxin receptor is created when domain 4 binds to the wild-type receptor (CMG2 or TEM8). Domain 4's N682 and D683 binding residues in particular are crucial for PA binding. Domain 2 of PA interacts with certain regions of the VWA region to facilitate receptor binding, which is primarily mediated by domain 4 of PA. The insertion loop of PA as well as the adjacent 22 and 23 -strands cannot be rearranged by binding to the 340-348 loop of PA. We created a domain-2/domain-4 interface-stabilized version of PA to stop this premature prepore-to-pore conversion. To do this, we genetically fused the C-terminus of PAwt with the 19.5-kDa sANTXR domain of CMG2 (residues 40-217, C175A). The precise orientation and functional interaction of the fusion partners are made possible by a lengthy (G4S)5 linker that connects PAwt and sANTXR and has a length of about 88. The covalent linker significantly boosts the local effective concentration of sANTXR, which when combined with the PA-binding domain's strong affinity is predicted to significantly lessen any unintended consequences. We created the PA mutant construct PAm-sANTXR-Ac2 with the mutations N682A and D683A (Additional file 1: Figure S1), which should prevent binding of PAm and sANTXR and thus have no stabilising interaction. This was done to ensure that the stabilising interaction is actually caused by the functional interaction of PA with the wild-type receptor domain. We also created a control variation with a very short linker between PAwt and the sANTXR domain, which restrained the sANTXR domain to an orientation that precluded steric binding of PAwt to sANTXR. A functional reliance between the stabilising interaction and prepore-to-pore conversion was investigated when these constructs were compared.

## Conclusions

An intelligently created new PA variant, PAwt-sANTXR-Ac2, significantly reduced the harmful effect that first prevented the retargeted delivery system from being improved further. The mechanism was improved as seen by increased cargo protein absorption overall and cytosolic delivery. We have demonstrated the increase using DARPins as an example, but the system is capable of delivering various proteins that can pass through the PA pore. We now hope for a wider spectrum of applications with appropriate intracellular drug targets with this upgraded PA version.

### Declaration of competing interest

The authors reaffirm that they are not aware of any personal or financial conflicts that might have appeared to affect the research described in this paper.

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