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TITLE: Use of Synthetic Antibodies Targeted to the Jak/Stat Pathway in Breast Cancer

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**4. TITLE AND SUBTITLE**

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Introduction and Scope of Research: Our research objective is to develop a novel set of technologies that will target the Jak/Stat signaling cascade in breast cancer (1). These technologies, which involve a new class of affinity reagents and intramolecular delivery tools (2, 3), have the capability of identifying the most important nodes in this signaling pathway and ultimately inhibiting or modifying them to influence effects on breast cancer cell proliferation and death. The interplay between the Jak and Stat components in cytokine signaling has been an area of intense investigation (4, 5). However, although many of the molecular interactions that occur between them and with other signaling partners have been broadly implicated in breast cancer, they are poorly characterized because of a lack of appropriate experimental tools. Consequently, a host of basic questions remain to be answered. Our goal is to develop an experimental framework to sort out the most important interactions in the pathway and establish whether there is a specific Achilles Heel that can be exploited to attack breast cancers in innovative ways. As a long-term goal, we will utilize this information to develop novel synthetic antibody reagents that can be delivered with precision and potency to breast cancer cells.

Research Accomplishments

Aim 1- Generate synthetic antigen binders (sABs) to components of the prolactin receptor signaling network. This was reported in the previous progress report in detail; significant accomplishments are summarized below.

Aim 2- Test the inhibitory sABs in cell-based assays to evaluate their effectiveness as receptor antagonists. Our hypothesis is that sABs that block hormone binding will have an inhibitory effect on downstream signaling as measured by Stat activation. To assess this, we have tested four inhibitory sABs in cell based assays to measure inhibition of Stat5 activation (Figure 1).

Cells were incubated with increasing concentrations of each sAB, or a control sAB against bacterial maltose binding protein. The effect of the sABs on prolactin signaling was determined using a dual luciferase luminescence assay under the control a of phospho-Stat promoter (Figure 1A).

The results indicate that 3 sABs (A8, A9 and A10) significantly inhibit prolactin signaling in a concentration dependent manner.

Figure 1- sAB inhibition of prolactin signaling. A) Effects on cell signaling based on luciferase expression. B) Measurement of decrease in phospho-Stat5 caused by the four individual inhibitory sABs.
To further examine the effect of the sABs on prolactin receptor signaling, we determined the change in the phosphorylation levels of various factors using western blot. While the control sAB (Fab for MBP) has no effect on prolactin signaling, sABs A8, A9 and A10 strongly inhibit phosphorylation of Stat5 and ErK (Figure 1B). Again, sAB A4 shows no effect on downstream signaling of prolactin as was observed in the luciferase assay.

Additionally, we hypothesize that inhibiting the proline isomerase activity of cytoplasmic CypA will effectively turn off downstream signaling by eliminating the switch that ultimately leads to Jak activation. To test this, we have used the sAB pipeline to generate a family of high affinity binders that are specifically targeted to block the active site of CypA and block binding of cyclosporine. Four inhibitory sABs have been evaluated using surface plasmon resonance to establish their binding affinities to CypA (Table 1). The affinities range from 2 nM to 12 nM.

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Table 1. SPR analysis of binding affinity of selected FABs. Purified sABs were injected over Sensor Chip NTA where 6His-tag CypA was immobilized on the surface. The equilibrium constant Kd was used to measure the binding affinity of two components.

A future goal is to use our receptor mediated delivery technology to introduce an inhibitory sAB into live cells to assess the consequence of selectively blocking CypA on prolactin signaling.

**Key Research Accomplishments**

- Used phage display to select sABs that act as inhibitors to CypA and CypB (previously reported).
- Generated and characterized a sAB against the PRLr-ECD and showed it was a potent inhibitor against PRL binding to the ECD (previously reported).
- Determined the X-ray crystal structure of the sAB-PRLr-ECD crystal structure at 2.8Å resolution (new).
- Established that sAB binding interferes with PRL binding through an allosteric mechanism (new).
- Assess the effects of CypA inhibition on prolactin signaling (new).

**Reportable Outcomes**

**Publications**


**Conclusion**

The reagents that are being generated are of exceptional importance to both basic and translational science in that they will provide a means of delivering inhibitory probes intracellularly into viable cells. The generation of sABs as outlined above has been successful and is following the timeline identified in the SOW.

**References**


**Appendicies** – Attached paper.

**Supporting Data** – None.
An engineered substance P variant for receptor-mediated delivery of synthetic antibodies into tumor cells

Shahir S. Rizk, Anna Luchniak, Serdar Uysal, Crista M. Brawley, Ronald S. Rock, and Anthony A. Kossiakoff

We have developed and tested a robust delivery method for the transport of proteins to the cytoplasm of mammalian cells without compromising the integrity of the cell membrane. This receptor-mediated delivery (RMD) technology utilizes a variant of substance P (SP), a neuropeptide that is rapidly internalized upon interaction with the neurokinin-1 receptor (NK1R). Cargos in the form of synthetic antibody fragments (sABs) were conjugated to the engineered SP variant (SPv) and efficiently internalized by NK1R-expressing cells. The sABs used here were generated to bind specific conformational forms of actin. The internalized proteins appear to escape the endosome and retain their binding activity within the cells as demonstrated by co-localization with the actin cytoskeleton. Further, since the NK1R is over-expressed in many cancers, SPv-mediated delivery provides a highly specific method for therapeutic utilization of affinity reagents targeting intracellular processes in diseased tissue.
Specific Delivery of Actin-Binding sABs to NK1R-expressing Cells. To demonstrate the ability of SPv to deliver fully-functional protein cargo, we chose a set of synthetic antibody fragments (sABs) engineered by phage display selection to bind distinct conformational forms of actin filaments. Characterization of the in vitro actin-binding properties of 3 sABs (sAB-27, sAB-4, and sAB-19) shows that each sAB exhibits binding to the actin filaments. Electron microscopy imaging indicates that sAB-27 reconfigures the overall structure of actin by inducing filament bundling (Fig. 3A). In contrast, sAB-4 was shown to induce rapid severing of the actin filaments by total internal reflection fluorescence (TIRF) microscopy (Fig. 3B). Finally, sAB-19 binds to the end of the filaments without disrupting the overall actin structure (Fig. 3C). To conjugate the sABs to SPv, a cysteine mutation was introduced at position 121 of the heavy chain of each sAB. This position is solvent accessible and lies far from the antigen binding site, allowing conjugation to the SPv variant via the maleimide moiety without interfering with actin binding. Before attachment to SPv, each sAB was fluorescently labeled via surface lysines using an amine reactive fluorophore to facilitate visualization by confocal microscopy.

As the structure and location of intracellular actin are readily identifiable by phalloidin staining, the ability of SPv to deliver functional sABs can be rigorously tested by the ability of the internalized sABs to disrupt or localize with actin filaments. U87 cells were incubated for 2 h with 20 nM SPv conjugated to sAB-4, sAB-27, or sAB-19. The cells were then fixed, stained with phalloidin, and analyzed using confocal microscopy. A z-series of cells treated with the sAB-SPv conjugates confirms internalization and localization of the conjugate with actin filaments (Movie S1). Additional analysis of the confocal microscopy images indicates that the SPv-attached sABs internalized by U87 cells retain their in vitro properties by co-localization or reorganization of the filamentous actin cytoskeleton.
SPv-sAB-27(Fig. 4B), suggesting that the SPv-NK1R system may be useful in the treatment of several cancers.

Quantitative Analysis of SPv-mediated Internalization of sAB-27. To determine the efficiency of SPv-mediated delivery of proteins, U87 cells were incubated with increasing concentrations of a cy5-labeled SPv sAB-27 conjugate for 24 h in a 96-well plate. The cells were extensively washed and residual fluorescence was determined, reflecting the amount of internalized conjugate. The results show a correlation between the amount of conjugate added and the concentration of the internalized conjugate that fits a hyperbolic response (Fig. 5). Addition of as little as 2 pmol of the conjugate results in the retention of approximately 0.15 pmol over roughly 5,000 cells in each well. Taking into account the average volume of U87 cells (500–1,000 μm³), we estimate that the intracellular concentration of the conjugate is in the mid-micromolar range (30–60 μM). This indicates that even a
small fraction (~1%) of the conjugate escaping the endosome would be comparable to the concentrations used for the in vitro studies, and would therefore, be sufficient to elicit a similar effect. It is important to note that the fit suggests an upper limit to the ability of US7 cells to internalize the SPv-conjugates with saturation at high concentrations of added conjugate. This apparent limit may be due to downregulation of the NK1R upon internalization of as a result of SPv uptake.

Discussion

We have developed an efficient and robust methodology that facilitates the delivery of protein cargos into the cytoplasm of live cells. The platform for our approach has been built such that it can be ported to the research community and used “out of the box.” The method utilizes the receptor-mediated internalization of SPv as an efficient transmembrane delivery vehicle, circumventing many of the problems associated with traditional transfection approaches, such as cytotoxicity. While ligand-induced receptor internalization is a ubiquitous mechanism of receptor regulation, the SP-NK1R system offers several distinct advantages over other systems. SP is a short peptide with subnanomolar affinity to the NK1R. Because only the C-terminal portion of SP is required for binding to the receptor, a wide variety of cargos can be linked to the N terminus.

A crucial feature of this RMD system is that enough of the SPv conjugates appear to readily escape the endosome without loss of function. We have tested other ligand-receptor systems that are efficiently internalized, but in many cases the cargo appears to remain trapped in the endosome and are thus biologically inactive. Although it has been suggested that SP is quickly degraded after internalization (16), it is not clear why SPv-conjugated cargos escape the endosomal machinery more efficiently than in other systems. It is possible that by linking cargo to SP the peptide is sufficiently modified in character that its fate is effectively altered. How and why this occurs is beyond the scope of this study. However, essentially all protein-based cargo that we have conjugated to SPv have been successfully delivered to the cytoplasm without loss of function. In complementary studies, we have determined that our engineered SPv can deliver a wide variety of bioactive cargo including nucleic acid-based molecules such as siRNA, shRNA, and DNA, as well as imaging agents and proteins up to at least 400 kDa. While the delivery of RNA/DNA molecules appears to be promising, further work is needed to rigorously profile their bioactivity in live cells.

Combining our SPv delivery technology with the ability to introduce functional sABs into live cells could be a transformative advancement for studying signaling pathways and other intracellular functions. In extensive studies, we have determined that phage display-library-sorting protocols can be designed to produce high affinity sABs that recognize a specific protein surface, a particular conformational state or a multicomponent complex. These attributes, combined with the ability to fine-tune the affinity to the target, result in a class of “designer” affinity reagents that can be programmed to carry out a wide range of directed functions within the cell following internalization. Examples of this include the sAB engineered against different forms of actin described here. Importantly, we have observed that sABs retain their function within the cytoplasm for at least several days after internalization. Thus, sABs engineered for live cell imaging, or inhibition or activation of specific functions in signaling pathways will have an extended functional lifetime in a live cell system.

Another notable feature of the system is its exquisite specificity for targeting certain types of cancer cells. We have shown that internalization of SPv is restricted to cells expressing the NK1R. This receptor is overexpressed in many types of tumors and primary cancers, including breast carcinomas, adenocarcinomas of the colon, astrocytomas, and glioblastomas (17). This ability to discriminate between cancer cells expressing the NK1R and normal cells suggests that receptor-mediated delivery using SPv as a Trojan Horse for bioactive cargo may have great therapeutic potential. Further, the specific delivery of sABs directed toward cytoplasmic targets within tumor cells potentially changes the paradigm for antibody-based therapies. These therapies may no longer be limited to the current extracellular targets. Thus, using sABs designed to inhibit intracellular signaling nodes, the possibility certainly exists for focusing future antibody therapies toward a much richer set of cancer targets.

Methods

Peptide Synthesis of SPv Variants. All protected amino acids and resin for peptide synthesis were purchased from Peptides International, solvents from Fisher, salts and buffers from Sigma Aldrich. SPv-maleimide synthesis was carried out manually by standard T-Boc methods using p-methyl-benzhydramine resin to produce a C-terminal amide after HF cleavage. 6-Maleimidohexanoic acid (Sigma) was used to introduce an N-terminal maleimide with a 6-carbon linker. An additional variant of SP was synthesized that lacks the maleimide moiety but includes a cysteine mutation at position 3 for attachment of thiol-reactive Alexa-555 and was used as a positive control for NK1 receptor-mediated internalization. The peptides were cleaved from the resin using HF and extracted in 50% acetonitrile plus 0.1% trifluoroacetic acid aqueous solution for lyophilization. The purity of the crude peptides was determined using an analytical C-18 reversed-phase column on a Shimatzu 10A-vp and their masses were confirmed by MALDI MS.

Fluorescence Labeling and SPv Conjugation to sABs. Actin-binding sABs were engineered by phase display and purified as described (22). Each sAB contained a C-terminal 6-his tag and a cysteine mutation at position Ala-121 of the heavy chain. The purified sABs were diazylized into 50 mM sodium borate buffer, pH 7.4, for fluorescent conjugation. NHS-ester fluorophores (Cy3 or Cy5; GE Healthcare, Alexa-488 or Alexa-647; Invitrogen) were added to each sAB in a 5-fold excess from a 10 mM stock solution in DMSO. Reactions were carried out at room temperature for 1 h, followed by gel filtration using a PD-10 column (GE Healthcare) equilibrated with 20 mM Mops, 100 mM NaCl, pH 6.9 to remove excess fluorophore. The fluorescently-labeled sABs were reacted with a 10-fold excess of SPv-maleimide from a 5 mM stock in DMSO. The reaction was carried out overnight at 4 °C in 20 mM Mops, 100 mM NaCl, pH 6.9, followed by gel filtration using a PD-10 column to remove excess SPv.

TIRF Microscopy. Images were collected with a custom-built total internal reflection fluorescence microscope. A 100 ×, 1.45 NA objective (Olympus) and an EMCCD camera (Ixon, Andor Technologies) were used (SI Methods).

Electron Microscopy. Electron Micrographs were obtained on a FEI Tecnai F30 transmission electron microscope (SI Methods).

Cell Lines, Western Blots, and Transfection. See SI Methods.

Confocal Microscopy. For each sample, 10⁴ U87 cells were seeded on an 18 mm cover-slip in MEM with 10% FBS overnight in a 12-well plate, then starred in MEM with 0.1% BSA for 3 h. The reagent (SPv-sAB conjugate, SP-Fluorophore, or sAB only) was filter-sterilized, diluted to the desired final concentration in MEM with 0.1% BSA and added directly to the live cells in each well for 2 h. Untreated cells or cells treated with sAB only served as a negative control. Following incubation with each reagent, the cells were washed 3 times with PBS, fixed in 4% paraformaldehyde, permeabilized in TBST and blocked with 10% normal goat serum for 1 h. Actin filaments were stained with FITC-phalloidin (Invitrogen, 1:250 dilution), or TMR-phalloidin (Sigma-Aldrich, 1:3,000 dilution) for 1 h at room temperature. Nuclei were stained with either DAPI (10 mg/mL) or Hoechst reagent (Invitrogen, 1:2,000) for 5 min at room temperature, and samples were mounted using ProLong Gold reagent (Invitrogen). Confocal microscopy images were collected using a Leica SP2 confocal microscope with FITC and Alexa-488 excited by a 488 nm laser, TMR and Cy3 by a 543 nm laser, and Alexa 574 and Cy5 by a 633 laser. Multicolor images were overlaid using the ImageJ software.

MTS proliferation assay. CellTiter 96 AQueous assay (Promega) was used to determine cell viability (see SI Methods).

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