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TITLE: Rapid Generation and Screening of Novel Bi-Specific Single-Chain FV Molecules Capable of Inhibiting the Growth of Breast Cancer

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Antibodies that perturb signal transduction of cancer cells have demonstrated significant utility in the treatment of breast cancer and lymphoma. As signal transduction in the Epidermal Growth Factor Receptor (EGFR) family (EGFR, HER2, HER3, HER4) involves ligand binding and subsequent heterodimerization of two members, the most potent monoclonal antibody (MAb)-based agent would likely be one that also mediates a similar crosslinking event. ***The fundamental hypothesis underlying this Concept Award Project was that signal transduction through components of the EGFR family could be manipulated through the construction of novel bispecific antibodies that engage multiple epitopes of this family.*** The goals of this proposal were to develop a novel, rapid methodology to create bispecific single-chain Fv (bs-scFv) molecules using molecular shuffling of two large groups of scFv (libraries) specific for HER2 and HER3 and to perform preliminary evaluations of the in vitro specificity and anti-tumor effects against cells that over express both target antigens.

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Introduction

Antibodies that perturb signal transduction of cancer cells have demonstrated significant utility in the treatment of breast cancer and lymphoma. As signal transduction in the Epidermal Growth Factor Receptor (EGFR) family (EGFR, HER2, HER3, HER4) involves ligand binding and subsequent heterodimerization of two members, the most potent monoclonal antibody (MAb)-based agent would likely be one that also mediates a similar crosslinking event. ***The fundamental hypothesis underlying this Concept Award Project was that signal transduction through components of the EGFR family could be manipulated through the construction of novel bispecific antibodies that engage multiple epitopes of this family.*** The goals of this proposal were to develop a novel, rapid methodology to create bispecific single-chain Fv (bs-scFv) molecules using molecular shuffling of two large groups of scFv (libraries) specific for HER2 and HER3 and to perform preliminary evaluations of the in vitro specificity and anti-tumor effects against cells that over express both target antigens.

Body

Research Accomplishments:

Task 1. Isolate scFv clones from a large human scFv library that are reactive with HER3. We used human HER3 extracellular domain (ECD) as a target to pan a phage display library. A number of scFv were isolated that bind to HER3 (Table 1). PCR fingerprinting revealed that 6 contained unique sequences. Note: while it was not directly identified in the scope of the proposal, we also isolated scFv molecules that bound to HER4 ECD (10 unique clones) and EGFR ECD (33 unique clones).

Table 1. Properties of scFv sublibraries.

Antigen	# of scFv libraries screened	# Unique scFv clones in sublibrary
HER2	2	2*
HER3	1	6
HER4	1	10
EGFR	3	33

*Note, 75 affinity mutants ranging in affinity from the mM to pM level have been created from one of these clones

Task 2. Create bs-scFv molecules using scFv shuffling. A unique peptide spacer with the amino acid sequence "NSGAGTSGSGASGEGSGSKL" was designed that would impart flexibility and resistance to serum proteases. Based upon incompatible restriction sites between the scFv clones, scFv shuffling techniques required extensive sight-directed mutagenesis on almost every clone to alter restriction sites. This effort proved to be be counter productive to the goal of speeding up the partnering and screening processes. We have recently initiated a new method of crosslinking pairs of scFv using a monoclonal antibody directed against a tag of the carboxy terminus. It is our belief that this will accomplish the same goal as the scFv shuffling would have – to identify optimal pairs of scFv for bs-scFv production. In the meantime, we selected a few anti-HER3 and anti-HER4 scFv molecules and used these together with the peptide spacer (shown above) and our best anti-HER2 scFv (ML3.9) to create a candidate bs-scFv that were screened for in

vitro specificity and efficacy. As a result of this effort, we have now created two bs-scFv that are specific for HER2 and HER3 (ALM and FLM), two bs-scFv that are specific for HER2 and HER4 (BLM and ELM), two homodimeric scFv that are specific for HER3 (ALA and FLF), one homodimeric scFv that is specific for HER2 (MLM) and one heterodimeric scFv that is specific for two epitopes on HER3 (FLM).

Task 3. Evaluate the specificity of the bs-scFv molecules for both target antigens. The specificity of the first bs-scFv, ALM, for HER2 and HER3 ECDs was evaluated by surface plasmon resonance on the BIAcore instrument. It was found to be capable of binding to HER2 ECD, HER3 ECD and of forming a complex between HER2 and HER3 ECDs in a sandwich assay (Figures 1-3). The specificity of BLM for HER2 and HER4 was similarly evaluated (Figure 4).

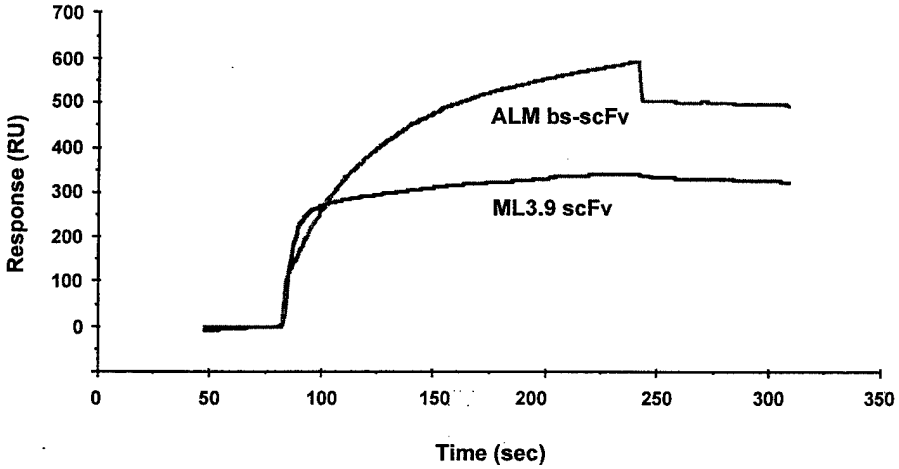


Figure 1. ALM (anti-HER2/HER3 bs-scFv) binding to HER2 ECD compared with the binding of the parent anti-HER2 scFv, ML3.9.

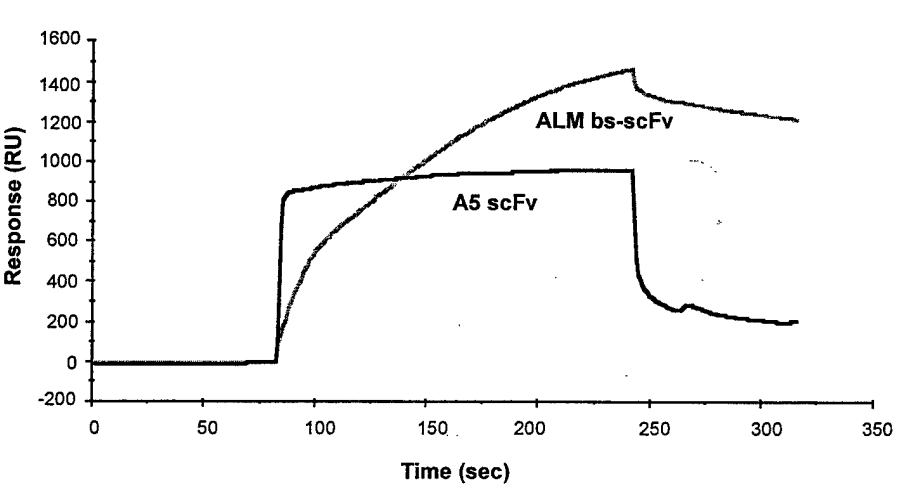


Figure 2. ALM (anti-HER2/HER3 bs-scFv) binding to HER3 ECD compared with the binding of the parent anti-HER3 scFv, A5.

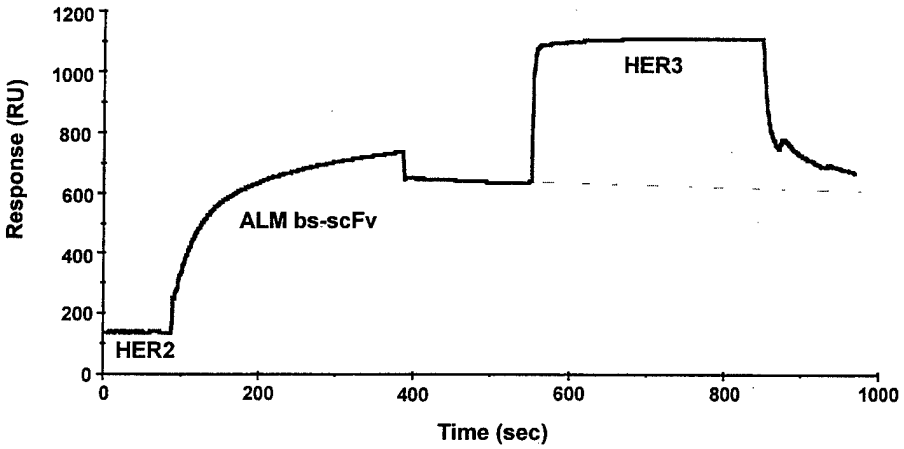
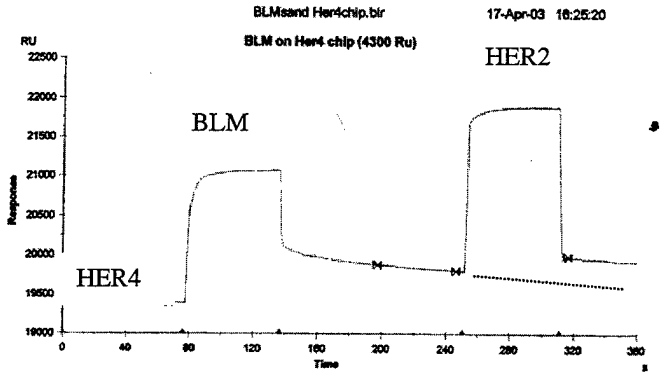


Figure 3. ALM (anti-HER2/HER3 bs-scFv) binding to immobilized HER2 ECD followed by HER3 ECD in solution in a sandwich assay.

Figure 4. BIAcore sensogram trace of the BLM anti-HER2/HER4 bs-scFv molecule demonstrates bispecific binding. BLM binds to HER4 that is immobilized on the chip and then binds the subsequently applied HER2. An extrapolation of the BLM dissociation (dotted line) reveals bispecific binding.



Task 4. Evaluate the function of the bs-scFvs. The functional consequences of the binding of first bs-scFv, ALM, to cells that overexpress HER2 and HER3 was evaluated in MTT assays, a clonogenicity assay and by flow cytometry. In a five day MTT assay, which is a measure of impact on cellular proliferation, ALM mediated a similar degree of growth inhibition of human breast tumor BT-474 cells as did Herceptin – a clinically approved MAb that targets HER2 (Figure 5). Similar results were seen with the BLM (ant-HER2/HER4) bs-scFv (Figure 6). In a 17 day clonogenicity assay, ALM incubated with BT-474 breast cancer cells at a roughly equimolar concentration to the amount of cell-surface HER2 lead to approximately a 50% reduction in tumor cell survival (Figure

7). Increasing the quantity lead to a decrease in efficacy. Finally, the impact of ALM on HER2 and HER3 expression on BT-474 cells was determined by flow cytometry. It was found that 1.6 ug/ml ALM incubated with 1.6 x 10^6 BT-474 cells resulted in a reduction of cell-surface HER2 after 4 hr @ 37°C and a reduction in cell surface HER3 after 48 hr @ 37°C (Figure 8).

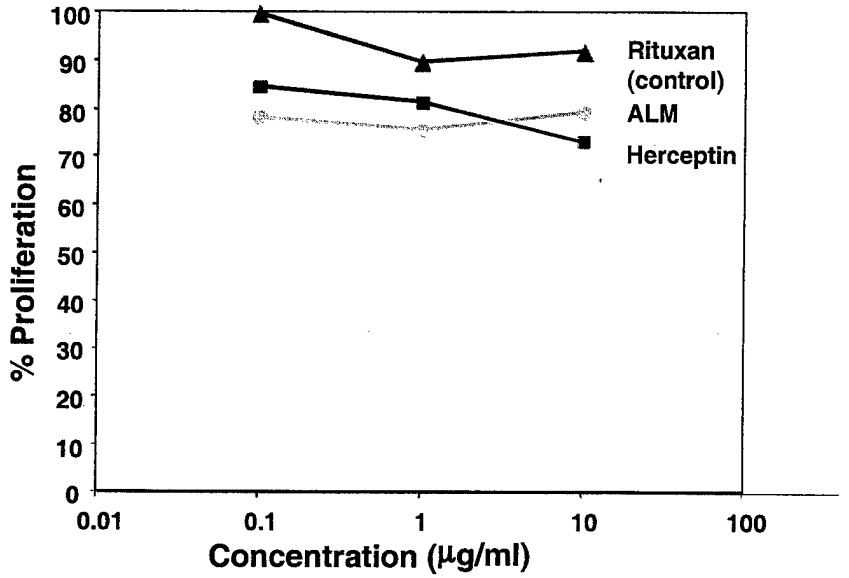
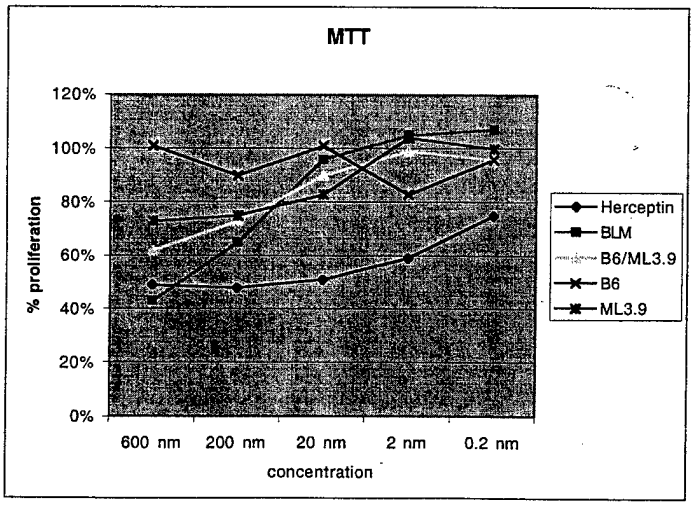


Figure 5. In an MTT assay, ALM has a similar anti-proliferative effect on human breast tumor BT-474 cells as does Herceptin. Rituxan is included as a negative control.

Figure 6. Representative cell proliferation (MTT) assay, using BT-474 breast cancer cell line, demonstrates that the growth inhibition properties of bs-scFv BLM (anti-HER4/anti-HER2) are superior to each scFv alone (B6 anti-HER4 or ML3.9 anti-HER2) or the combination of both scFv (B6/ML3.9) and are equal to Herceptin (positive control) at the highest dose.



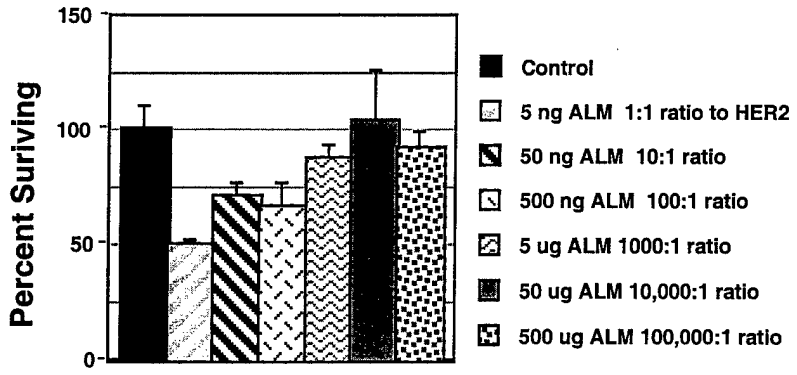


Figure 7. Results of a 17 day clonogenicity assay using increasing concentrations of ALM with BT-474 cells. Ratios of ALM to HER2/*neu* are indicated, n=3 plates/concentration.

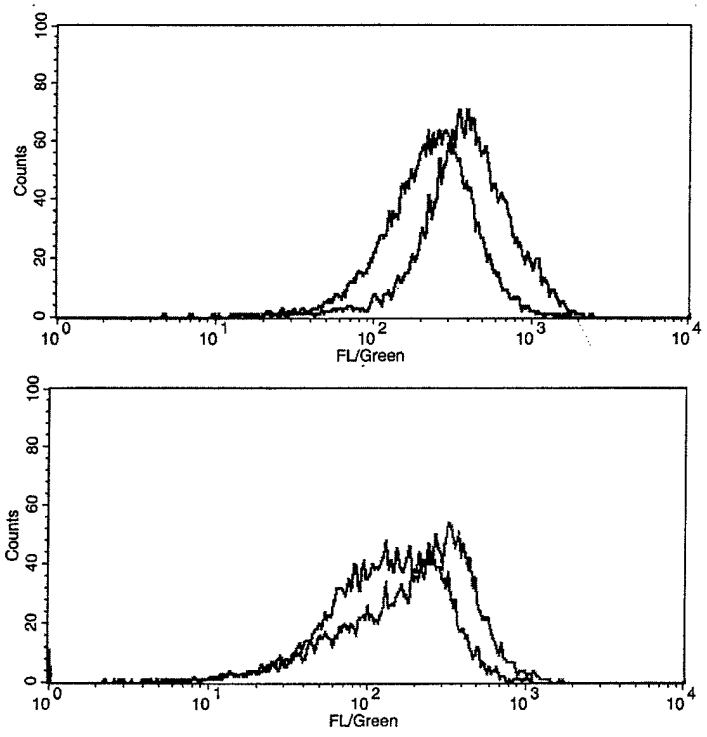


Figure 8. Impact of ALM on the expression of HER2 at 4 hrs (top) and HER3 at 48 hrs (bottom) on the surface of BT-474 cells. The left-hand peaks on each plot are with ALM, the right-hand peaks are without ALM. 1.6 ug/ml ALM incubated with 1.6 x 10^6 BT-474 cells, HER2 detected with 520C9 MAb and HER3 detected with AB4 (Neomarkers).

Key Research Accomplishments

- Isolated scFv from a human phage display library that are specific for HER3, HER4 and EGFR. 6 unique clones that bind to HER3, 10 unique clones that bind to HER4 and 33 unique clones that bind to EGFR.
- Developed a novel peptide spacer that is expected to be protease resistant for use in linking the scFv into a bispecific format (Spacer sequence = NSGAGTSGS GASGEGSGSKL)
- Created the following scFv-based molecules; two bs-scFv that are specific for HER2 and HER3 (ALM and FLM), two that are specific for HER2 and HER4 (BLM and ELM), two homodimeric scFv that are specific for HER3 (ALA and FLF), one homodimeric scFv that is specific for HER2 (MLM) and one heterodimeric scFv that is specific for two epitopes on HER3 (FLM).
- Evaluated the impact of the lead bs-scFv molecule, ALM, on the in vitro growth properties of the human breast tumor cell line, BT-474, that overexpresses HER2 and HER3.
  - In an MTT assay evaluating the impact on cell proliferation, a five day incubation with ALM, Herceptin (positive control) or Rituxan (negative control) lead to comparable inhibition of proliferation with ALM and Herceptin.
  - In a clonogenicity assay, a 17 day incubation with 5 ng to 500 ug concentrations of ALM (ALM to cell surface HER2 ratios of 1:1 to 100,000:1) lead to approximately 50% reduction in cell viability in the group treated with the lowest concentration of ALM. Interestingly, as the concentration was increased, the impact on survival decreased.
  - Have determined by flow cytometry that incubation of BT-474 cells with ALM leads to decreases in the cell surface expression of HER2 and HER3.
- Evaluated the impact of a second bs-scFv molecule, BLM, on the in vitro growth properties of the human breast tumor cell line, BT-474, that overexpresses HER2 and HER4.
  - In an MTT assay evaluating the impact on cell proliferation, a five day incubation with ALM, Herceptin (positive control) or Rituxan (negative control) lead to comparable inhibition of proliferation with BLM and Herceptin.

Reportable Outcomes

- American Association for Cancer Research abstract. #4810. Production and Evaluation of Bispecific single-chain Fv molecules that target HER2/neu and HER3. E. Horak, L. Shahied, C. Shaller, A. Tesfaye, H. Simmons, R. Alpaugh, N. Greer, T. Heitner, J. Garrison, J. Marks, L. Weiner and G. Adams.Proc. AACR. 43:971, 2002.
- Presentation at "AntibOZ", An International Forum: Predicting the Next Wave of Protein-Based Therapies and Immunodiagnostics. Heron Island, Queensland Australia. April 8-12th, 2002.

- Presentation at Keystone Meeting Feb, 2003 in Banff Canada. "Targeting the Epidermal Growth Factor Receptor Family with Heterodimeric Single-chain FV molecules. Eva M Horak, Matthew K Robinson, Tara Heitner, Jennifer L Garrison, Lillian S Shahied, Calvin C Shaller, Abohawariat Tesfaye, Heidi H Simmons, R Katherine Alpaugh, Nathaniel B Greer, James D Marks, Louis M Weiner and Gregory P Adams.
- Patent applied for April 5, 2002, Serial No. 60/370,276.

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Conclusions
The generation of a scFv shuffling system for creating multiple bs-scFv partners was more difficult than expected. While progress has been made in this area, we have proceeded to make two bs-scFv molecules that target HER2 and HER3, two that target HER2 and HER4 and homodimeric controls that target HER2 or HER3. The lead molecule, ALM, has been evaluated for specificity and efficacy in vitro. We have found that it binds specifically to both targets and mediates a decrease in cell surface expression of HER2 and HER3 and leads to a reduction in cell survival and proliferation. We plan to evaluate the in vivo efficacy of ALM in immunodeficient mice bearing s.c. human breast cancer tumors. If ALM shows a similar effect in vivo, we will plan on initiating clinical trials as soon as possible.

References
N/A