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FOREWORD

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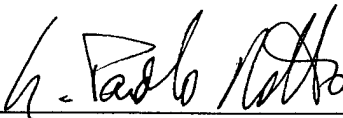

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Introduction

Macromolecular drug carriers provide one of the most promising approaches to improve delivery of therapeutic and diagnostic drugs to cancer cells. A very significant achievement in this area relates to the recent development of "long-circulating" macromolecular and colloidal preparations (polymers, micelles, liposomes, etc.). Clearance of these compounds from the blood stream and uptake by the reticuloendothelial system (RES) are very slow. As a consequence, macromolecular drug carriers circulate long enough to extravasate into tumors via "leaky" endothelium, and accumulate at these sites as a result of deficient lymphatic drainage, non-specific tissue binding and spontaneous endocytosis.

Although long circulating drug carriers do accumulate in solid tumors, they do not specifically bind cancer cells. Thus, the microdistribution of the drug within the tumor tissue may be suboptimal. Also, the drug complex can partially return to the blood stream by a reverse of the extravasation process through the leaky endothelium, or lymphatic drainage, or both. These limitations have two major consequences: 1) therapeutic drug delivery has limited efficacy, so that the therapeutic index remains relatively low; 2) diagnostic imaging agents for tumor staging have low specificity and, for small metastases, low sensitivity. Substantial improvements could be achieved by the association of long-circulating drug carriers with "vector molecules" capable of binding specifically to cancer cells. These molecules would (a) direct carrier binding specifically to cancer cells, minimizing nonspecific uptake by the surrounding tissue; and (b) prevent the carrier from exiting the tumor, and thus maintaining higher local drug concentrations. Previous attempts to develop cancer-specific vector molecules were based mostly on antibodies against tumor-specific antigens. This approach, however, has several limitations, related to the large size of the antibody molecules, and the fact that cancer-specific antigens are usually expressed at low concentrations, and only in limited subsets of cancer cells.

Our proposal is based on the idea of using a genetic selection/screening technique to identify peptides that selectively recognize breast cancer cells (as opposed to normal cells of the same or different type). Such cancer-specific peptides will then be coupled to fleximer-based long-circulating drug carriers to confer upon these compounds the desired specificity.

Body of Work

1) Assumptions, Experimental Approach and Results.

One frequent genetic alteration connected with breast cancer development is amplification and/or overexpression of the Erb2 oncogene. In order to optimize our selection/screening procedure for breast-cancer specific peptides, we decided at first to focus on the identification of peptides that can selectively bind to the extracellular portion of the Erb2 protein. We were prompted by several reasons. First, by knowing what the specific target of the selected peptides would be, it would be possible to measure affinity of binding of the peptide-expressing phage, as well as of the biochemically synthesized peptide, either free in solution or coupled to a macromolecular carrier. This in turn would have allowed us to optimize conditions for additional studies with peptides binding to unidentified targets. Second, we made a collaborative arrangement with Dr. Kermit Carraway at Harvard Medical School, who provided us with SF9 insect cells overexpressing the Erb2 protein in a functional state on their surface. Use of normal SF9 cells for control experiments would eliminate all background problems due to binding of endogenous Erb2 which is normally expressed on the surface of mammalian cells. More recently, Dr. Carraway has provided us with the purified extracellular domain of the Erb2 protein produced in a soluble form, that we have started to use as an alternative target for our phage panning/selection assays (as described in detail further below).

Three phage display libraries have been used for these studies, that were purchased from New England Biolabs. Each of these libraries was subjected to several cycles of binding, saline washing, elution at acidic pH, and amplification using (a) Erb2-overexpressing versus control SF9 cells; (b) Erb2-overexpressing versus control NIH-3T3 cells; (c) Erb2 overexpressing versus non-expressing mammary carcinoma cell lines. The phage species recovered at the end of these cycles were cloned, purified and tested for selectivity of binding to Erb2 overexpressing versus control cells.

In the first year of our work we screened a library of phages expressing a random stretch of 7 amino acids at the amino terminus of the phage gene III protein, and a second library with a random 12 amino acid sequence inserted at the same location. As described in detail in our previous progress report, our screening attempts yielded unsatisfactory results. We thus switched to a third library of phages that express a 7 amino acid random sequence flanked by a cysteine at both ends, and inserted at the amino terminus of the gene III protein. After only two rounds of screening, more than 80% of the phages that were examined expressed the same peptide sequence. This peptide sequence was not found in 20 random phages that were examined from the initial library. The sequence of this peptide is:

Cys-Pro-Asn-Pro-Asn-Asn-Lys-Asn-Cys

Computer simulation for the most thermodynamically-favourable configuration of the peptide pointed to a very interesting feature, namely that the peptide can be present in a cyclic form:

-Cys-Pro-Asn-Pro-Asn-Asn-Lys-Asn-Cys-
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which can be dramatically stabilized when complexed with calcium ion (Figure 1). This finding raised the possibility that the peptide, either in its phage-bound or chemically synthesized form, binds to Erb2-expressing cells in a calcium-dependent manner. However, no such calcium-dependence was observed. Furthermore, the peptide expressed on the phage surface exhibited only a 4-7 fold increased specificity of binding on Erb2 expressing cells versus controls, and no such specificity was observed when the peptide was tested in a free soluble form or as a complex with a macromolecular carrier.

For this reason, we decided to abandon this peptide and look for an improved screening strategy that might enhance our chances of success. In particular, the main problem that we encountered in all our screening attempts, was the isolation of only a very limited number of phages expressing Erb2-binding peptides. Our priority at this point was that of isolating a much higher number of phages different expressing Erb2-binding peptides, so that we would have greater chances of identifying peptides that would maintain high affinity and specificity of binding even when tested after chemical synthesis. Thus, we devoted our subsequent efforts to the optimization of a phage panning selection using as target the purified extracellular domain of the ERb2 protein. Dr. Carraway was recently able to produce this domain of Erb2 in SF9 cells as a secreted soluble, form. The protein produced in this manner appears to retain much of its native configuration, as indicated by the fact that it is still recognized by antibodies that recognize extracellular Erb2 only under native conditions. Panning of phage libraries on the purified Erb2 instead of Erb2-expressing cells will have the significant advantage of eliminating the binding of phage to other membrane cellular proteins, and therefore should greatly increase our chances of obtaining a much larger collection of Erb2-binding phages. In our initial studies, we performed phage panning experiments with Petri dishes coated with the Erb2 protein. A blocking solution with BSA was used to prevent aspecific binding to plastic. However, these blocking conditions were too strong and need to be better optimized. Alternatively, the soluble Erb2 protein is produced as a his-tagged form, so that it will be possible to perform phage panning selection with the protein attached to beads rather than absorbed to dishes.

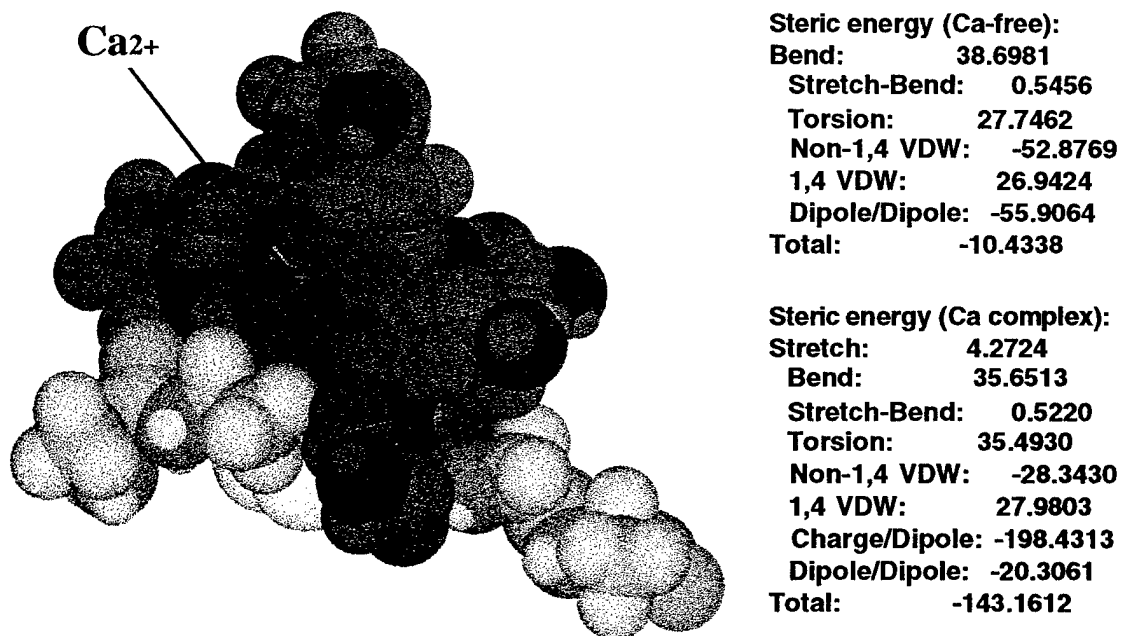


Figure 1. Cyclic form of Cys-Pro-Asn-Pro-Asn-Asn-Lys-Asn-Cys forms a potential metal-binding site (molecular simulation). Steric energies calculated based on MM2 model.

2) Methods : High titer ($\sim 10^{11}$ particles/ml) phagemid library preparations were preabsorbed in PBS to unfixed control cells, and allow to bind for 10'. Supernatant was removed and added to unfixed Erb2 overexpressing cells for 20' at room temperature. Non-bound virions were removed by repeated washes with saline solution, while binding virions were recovered with a low pH solution. After each cycle, recovered phages were amplified by growth into bacteria. The phages recovered at the end of these cycles of positive and negative selection were cloned, purified and retested for their specificity of binding to Erb2 expressing versus control cells, and the sequence of their random amino acid inserts was established. Similar panning experiments were also repeated with petri dishes to which the purified extracellular domain of the Erb2 protein was previously absorbed (using a protein concentration of 0.1mg/ml, in PBS). After coating with the Erb2 proteins, dishes were treated with a "blocking solution" with 0.1 mg/ml BSA, to prevent the aspecific binding of phage to plastic. Unfortunately, we found that under these conditions a significant number of phage bound aspecifically to BSA, so that no specific Erb2-binding phages could be recovered.

The complementary part of this work involved the synthesis and purification of a peptide analogous to the specific random peptide expressed by the Erb2-binding phages. The purified peptide was labeled with a radioactive or fluorescence moiety and tested for its specificity of binding to Erb2 overexpressing versus control cells. The same peptide was also coupled to a macromolecular drug carrier. The peptide comprising a terminal cystein (incorporated during peptide synthesis) was conjugated with S-thiopyridyl groups of the carrier. The latter were introduced into the carrier

structure via carrier conjugation with cystamine, with subsequent reduction of the S-S bridge followed by the thiol group reaction with dithiopyridine. The peptide coupled to the macromolecular carrier was tested on Erb2 overexpressing versus control cells as before.

3) Model carriers for drug targeting with cooperative vector molecules.

While testing phage display libraries for breast cancer-targeted peptides, we continued developing prototype cooperative carrier systems utilizing a previously developed model system based on N-formylpeptide - formylpeptide receptor interaction.

Peptides binding membrane markers of white blood cells, including the leukocyte N-formylpeptide receptor (FPR), have been found to accumulate in inflammatory regions as a result of association with white blood cells invading the inflammation site. This fact was utilized to study the behavior of cooperative polymer-based vectors comprising several peptide moieties, as compared to the analogous monomeric peptide. The objectives of this fragment of the study were: (i) prepare macromolecular preparations of varying length and comprising varying number of chemotactic peptide (N-formyl-Met-Leu-Phe-Lys) moieties per macromolecule length; (ii) determine the optimal range of peptide content; (iii) determine the molecule size range providing a most favorable blood clearance rate for agent accumulation in inflammatory areas; (iv) synthesize a model agent for inflammation scintigraphy comprising a g-emitting radionuclide and a fluorescent label; (v) characterize the biokinetics and imaging characteristics of the model preparation as compared to the radiolabeled peptide monomer.

Macromolecules comprising multiple f-Met-Leu-Phe-Lys (fMLFK) moieties and DTPA were synthesized using linear poly[hydroxymethyl-ethylene hydroxy-methyl-formal] (PHF) backbone. PHF, a biomimetic "stealth" polymer, is a base compound of Fleximer family. An aldehyde form of PHF with broad molecular weight distribution (10 to 150 kDa) was conjugated with cystamine (spacer precursor) and fMLFK in one stage, at various molar ratios, in the presence of cyanoborohydride. The conjugates were subsequently reduced with borohydride to transform the unused aldehyde into hydroxymethyl groups and S-S bridges to mercaptogroups. The latter were derivatized with DTPA anhydride and (in some preparations) with trace amounts of fluorescein-5-maleimide. The resultant polymers were purified by gel chromatography, fractionated by SEC HPLC, and labeled with ^{111}In via transchelation in citrate buffer. Control polymers containing DTPA but no peptide were prepared analogously. Radiolabeled peptide monomer was prepared via fMLFK derivatization with DTPA and labeling with ^{111}In .

Pilot biokinetics and biodistribution studies were performed in normal male CD rats (n=3 per preparation) to determine the optimal ranges of peptide content and molecular weight of the polymer. Conjugates with broad molecular weight distribution were fractionated by SEC HPLC to obtain fractions with MW=10, 50 and 80 kDa (MW distributions overlapped by ca. 30% by HPLC). For the preliminary testing, conjugates containing from 0.5 to 20 % fMLFK (mol/mol monomer) were prepared. Pilot studies showed that the optimal range by conjugate content is ca. 3-10%, and the optimal dose range, with regard to clearance rate, is > 0.1 mg/kg.

For the biodistribution studies in rabbit inflammation model, preparations containing 5% of each fMLFK and DTPA were synthesized. Non-overlapping 15 and 75 kDa fractions used in this experiment showed statistically significant difference in biokinetics. The preparations were labeled with ^{111}In and injected iv into 2.5 kg New Zealand rabbits, $n=4$ per group, 50-100 $\mu\text{Ci}/\text{animal}$ (0.5 mg/kg total substance). Animals were normal or bearing focal bacterial inflammation induced by inoculation of *E. Coli* (clinical isolate) in thigh muscle. Indium-labeled PHF-DTPA and monomeric DTPA-fMLFK were used as control preparations. Images were acquired over a 20 hr. period, followed by a biodistribution study. Biodistribution data showed that, compared to the monomer DTPA-fMLFK, renal accumulation was greatly reduced (by 81% and 88% for 70 and 15 kDa preparations respectively), while accumulation in the infected muscle was reduced by only 37% and 72%. Target to normal muscle ratios were 25 ± 10 and 14 ± 4 , compared to 33 ± 21 for the monomer. In blood, testes, adrenals and heart, accumulation was reduced by 40-50%. Hepatic and splenic depositions were reduced by 40% for the 15 kDa preparation, and increased by 50% for the 70 kDa one. In other tissues, label content did not significantly differ from the monomer. By 20 hr., accumulation of DTPA-PHF-fMLFK in the infected site resulted in clear delineation of the inflammation in all images. It has been found that the high molecular weight preparation accumulate in the inflammation, in part, as a result of non-specific vascular leakage/retention, analogous to that in tumors. Accumulation of the low molecular weight preparation in the inflammatory site was completely target-specific, i.e., entirely dependent on the action of the chemotactic peptide. Based on this observation, we hypothesize that small targeted polymers may be preferable as targeted carriers; this hypothesis will be further tested in the ongoing research.

Conclusions

Long circulating drug carriers accumulate in solid tumors, but do not specifically bind cancer cells. Thus, therapeutic drug delivery has limited efficacy and diagnostic imaging agents have relatively low specificity and, for small metastases, low sensitivity. Substantial improvements could be achieved by the association of long-circulating drug carriers with "vector molecules" capable of binding specifically to cancer cells. Our proposal is based on the idea of using a genetic selection/screening technique to identify peptides that selectively recognize breast cancer cells (as opposed to normal cells of the same or different type). Such cancer-specific peptides will then be coupled to fleximer-based long-circulating drug carriers to confer upon these compounds the desired specificity. In the first year of our work, we optimized conditions and screened three different phage display libraries for peptides that selectively bind to Erb2, an important cell surface receptor overexpressed in breast cancer cells. We confirmed that the phage display technology can be used to identify peptides that bind to proteins expressed on the surface of breast cancer cells. The challenge for the second year of our work was to identify peptides that retain specificity of binding when tested separately from the phage with which they were identified. Unfortunately, all peptides lost specificity of binding, when assayed independently from the phages, either in a free soluble form and/or coupled to a macromolecular carrier. As an alternative method to identify Erb2-binding peptides with higher selectivity of binding, we have undertaken phage panning experiments with the purified extracellular domain of the Erb2 protein produced in a soluble form. Work for the upcoming year will be aimed at optimizing our panning conditions for the purified Erb2, and for the selection of phages with high affinity and specificity of binding. At the same time, we will be producing large amounts of recombinant EGF and Heregulin 1, which are well known ligands of Erb2 when paired with the EGF receptor and Erb3, respectively. The recombinant EGF and Heregulin will then be coupled to macromolecular carriers for binding to Erb2 overexpressing breast carcinoma cells, as for the experiments that were originally designed for the Erb2-specific peptides.

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