Engineered Proteins for Visualizing and Treating Cancer

Jennifer R. Cochran, Ph.D.
Associate Professor of Bioengineering, and (by courtesy) Chemical Engineering
Stanford Cancer Institute
Stanford Schools of Engineering and Medicine, Stanford University

Introduction

Cancer is complex and its diagnosis and treatment can more effectively be tacked by teams of scientists, engineers, and clinicians whose expertise spans bench-to-bedside approaches. An emerging core philosophy applies understanding of molecular mechanisms underlying disease pathophysiology as design criteria towards developing safer and more efficacious tumor targeting agents (1). Armed with this knowledge, academic and industrial researchers are using a variety of approaches to create tailor-made proteins for applications in cancer imaging and therapy. These efforts leverage enabling tools and technologies, including methods for: 1) protein design and engineering, 2) biochemical and biophysical analyses, and 3) pre-clinical evaluation in animal models. Important goals of this work include providing fundamental insight into ligand-mediated cell surface receptor interactions that drive disease, and developing new protein-based drugs for translation to the clinic.

As the field of protein engineering evolved throughout the 1980s, engineered proteins soon surpassed recombinant versions of natural proteins as the major class of new therapeutics. The ability to customize the biochemical and biophysical properties of proteins to augment their clinical potential has presented new opportunities for the pharmaceutical industry. The market value of biopharmaceuticals is currently over $140 billion, exceeding three-quarters of the economies listed in the World Bank GDP rankings (2). Monoclonal antibodies used to treat cancer, rheumatoid arthritis, and cardiovascular and other diseases comprise a large share of

these efforts (3). In 2014, the US and European market included close to 50 monoclonal antibody drugs, a $75 billion market (4). In 2014-2015 alone, three of the top five cancer treatments were antibodies. Rituximab (Rituxan), Bevacizumab (Avastin), and Trastuzumab (Herceptin), all produced by Genentech / Roche, were the first, second, and fourth highest revenue-generating cancer drugs. The size of this market underscores both the clinical and economical importance of engineered proteins in the translational medicine space.

Current challenges of targeted cancer therapeutics include the need for more selective localization to tumors versus healthy tissue and improved delivery to brain tumors, which are protected by a restrictive blood-brain-barrier (5). Other challenges include acquired drug resistance that cannot be overcome due to dose limiting toxicity and lack of effective drugs to treat cancer once it has spread. Limitations of antibodies in addressing these and other challenges have motivated the development of alternative tumor targeting proteins that possess different molecular sizes and biophysical attributes, conferring alternate pharmacological properties (6). Below we describe some examples of engineered protein therapeutics developed by our research team that are modulating cancer in new and impactful ways.

An ultra-high affinity engineered protein therapeutic for treating metastatic disease.

Despite advances made over the past few decades in the development of targeted therapeutics, there is a lack of effective drugs available to treat cancers once they have spread, with 90% of all patients succumbing to metastatic disease. We teamed up with cancer biologist Amato Giaccia (Stanford Radiation Oncology) to address this challenge. In a number of human cancers, aberrant signaling through the Axl receptor tyrosine kinase has been demonstrated to drive metastasis (7),

confer therapeutic resistance (8), and promote disease progression (9). Additionally, Axl overexpression has been observed in multiple solid and hematological malignancies (10), with expression levels often correlating with disease stage and poor clinical prognosis (8, 11, 12). Ambiguity surrounding fundamental characteristics of Axl's interaction with its ligand, growth arrest specific 6 (Gas6), including its affinity and the mechanism of receptor activation, have hindered the development of effective Axl antagonists.

We used rational and combinatorial approaches to engineer an Axl 'decoy receptor' that binds Gas6 with high affinity and inhibits its function (13). Upon fusion to an antibody Fc domain, the engineered decoy receptor binds Gas6 with an affinity of ~400 femtomolar, placing it amongst the tightest protein-protein interactions found in nature. Crystallographic analysis of the co-complex structure showed that four mutations in Axl induced structural alterations in side chains across the Gas6/Axl binding interface and stabilized a conformational change on Gas6. The engineered decoy receptor effectively sequestered Gas6, allowing complete abrogation of Axl signaling. Moreover, Gas6 binding affinity was critical and correlative with the ability of decoy receptors to potently inhibit metastasis and disease progression. The engineered Axl decoy receptor inhibited >90% of metastatic nodules in two murine models of ovarian cancer compared to wild-type Axl (~50% inhibition), and demonstrated significant therapeutic efficacy compared to small molecule kinase inhibitors, with virtually no toxic side effects (13).

Using inspiration from nature to develop a novel class of tumor targeting agents. A major obstacle hindering the development of therapeutics that target the brain is the presence of the blood-brain-barrier (BBB), which prevents foreign particles and molecules from entering the

central nervous system. We recently demonstrated the promise of using engineered peptides, known as knottins, to specifically target brain tumors for applications including image-guided resection and targeted drug delivery (14). Knottins are unique peptides (30–50 amino acids) that contain a disulfide-bonded core which confers outstanding proteolytic resistance and thermal stability (15). Knottins are found in a wide variety of plants, animals, insects, and fungi, and carry out diverse functions such as ion channel inhibition, enzyme inactivation, and antimicrobial activity (16). In previous work, we used molecular engineering approaches to redirect a knottin found in squash seeds that normally functions as an enzyme inhibitor, to create an engineered knottin that binds tumor-associated receptors with high affinity (17). In collaboration with Zheng Cheng and Sanjiv Sam Gambhir (Stanford Radiology) we established these engineered peptides as a new class of molecular imaging agents for cancer (18). We then showed that intravenous injection of an engineered knottin, conjugated to a near infrared fluorescent dye molecule, targeted and illuminated intracranial brain tumors in animal models of medulloblastoma (collaboration with Matthew Scott, Stanford Developmental Biology, Genetics, and Gerald Grant, Stanford Neurosurgery) (14, 19). This collective work has been recently reviewed (20, 21).

Disulfide-rich peptides, including knottins, have generated great interest as drugs, as they have the potential to fill an important niche that lies between small molecules and protein biologics. Namely, they offer desirable drug-like properties of small molecules with the target-binding affinity and specificity of protein biologics. We postulated that if we could use the engineered knottin peptide to see tumors, then we could use it as a vehicle to deliver drugs to tumors, with a goal of minimizing toxic side effects of systemic chemotherapy. In one study, the engineered

knottin peptide was conjugated to the nucleoside analogue gemcitabine using a variety of linker strategies and an optimal candidate was shown to inhibit proliferation of breast, ovarian, pancreatic and brain tumor cells in vitro (22). Notably, this peptide-drug conjugate was shown to kill cells through a receptor-mediated internalization, and thus exhibited increased potency against pancreatic cells that acquired some resistance to treatment with gemcitabine alone. In a second study, the engineered peptide was fused to an antibody fragment (called Fc), conjugated to the tubulin inhibitor monomethyl-auristatin-F, and developed as a knottin-Fc-drug conjugate capable of inducing regression and prolonged survival in a flank glioblastoma model (23). These studies demonstrate the potential for further developing antibody alternatives for tumor targeting and drug delivery applications.

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